Regulatory DNA Elements Modulating Hypoxia-Inducible Erythropoietin Gene Expression

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Summary

Erythropoietin is the prime hormone regulating red blood cell homeostasis. The main organs producing Epo are the fetal liver and the adult kidney, but ectopic Epo expression can be found also in other organs, including the brain. In response to anemia or inspiratory hypoxia, Epo synthesis is transcriptionally induced by activation of the hypoxia-inducible factor (HIF) pathway, allowing the binding of HIF-2 to a hypoxia response element (HRE) within the *EPO* gene. The availability of liver-derived cell models as well as *in vivo* studies permitted the characterization of the Epo transcriptional regulation in the liver, leading to the finding that the 3' HRE is the regulatory element responsible for the induction of hepatic Epo expression in hypoxia. Reporter transgenic mouse models demonstrated that a region encompassing -14 to -6 kb upstream of the transcription start site (TSS) of *EPO* gene contains the renal regulatory enhancers involved in the regulation of renal Epo and, it has been termed kidney inducible element (KIE). Within the KIE, we previously identified by *in silico* analysis a novel 5' HRE located at -9.2 kb from the TSS of *EPO.* We showed that this 5' HRE is highly conserved among species and that drives exogenous reporter gene expression in hypoxia.

Because the liver-specific 3' HRE is dispensable for renal Epo regulation and a renal cell culture model was missing until recently, we investigated the contribution of the novel 5' HRE and the well-established 3' HRE by endogenous gene editing (CRISPR/Cas9) of both HREs in Kelly as well as Hep3B and HepG2 cells, capable of producing Epo in a hypoxia-inducible manner. We showed that while in hepatic cells the 3' but not the 5' HRE is required, in neuronal cells both the 5' and the 3' HREs contribute to regulate hypoxia-inducible endogenous Epo expression. Moreover, we identified two not yet reported HREs within the *EPO* promoter, termed pHRE1 and pHRE2, in line with a weak but significant promoter-driven reporter gene induction. While in hepatoma cells HIF interacted mainly with the distal 3' HRE, in neuronal cells, HIF bound most strongly the *EPO* promoter, weaker the 3' HRE and not at all the 5' HRE, suggesting that the 5' HRE acts in a non-canonical way by recruiting other transcription factors rather than HIF. We additionally generated 5'/3' double mutant Kelly cells and could show that both distal 5' and 3' HREs functionally cooperate with HIF binding to promoter HREs. These findings provide new insights into brain Epo regulation and they might be of help to recapitulate renal Epo regulation as renal Epo-producing (REP) cells show neuronal features.

Due to the lack of a renal cell culture model able to produce Epo in a hypoxia-inducible manner, the study of renal Epo transcriptional regulation has been constrained. Therefore, we generated a novel transgenic mouse model to isolate fresh primary REP cells and we established a new

method to culture both, primary cells and immortalized clonal cell lines, which we named fibroblastoid atypical interstitial kidney (FAIK) cells. By two simultaneous stimuli, hypoxia and tamoxifen, we were able to temporally and conditionally tag the REP cells with red fluorescence due to permanent activation of tdTomato reporter gene and negatively select the Cre^{ERT2} expressing cells through inactivation of the diphtheria toxin receptor (DTR) as non-Cre^{ERT2} expressing cells died in presence of diphtheria toxin. This mouse model is, thus, useful to generate primary REP culture and clonal REP cell lines that can be adopted as model to study hypoxic renal Epo regulation.

We also applied a novel chromogenic *in situ* hybridization method to visualize on a single cell level Epo mRNA molecules as well as other HIF target genes in neuroblastoma, hepatoma and FAIK cells. We provided initial evidence for an inherent cell-to-cell variability of the Epo hypoxic transcript pattern not observed with constitutively expressed transcripts.

This PhD work focussed on the transcriptional regulation of erythropoietin and on the DNA regulatory elements enhancing Epo expression in neuroblastoma and hepatoma cell lines. We could show that, in contrast to the liver, in the brain, Epo regulation occurs through a complex interplay between the distal 5' and 3' HREs and Epo promoter, demonstrating that several HREs can cooperate in oxygen-regulated gene expression in a cell-type specific manner.

1. Introduction

1.1 History of erythropoietin: from biochemistry to sport

Erythropoietin (Epo) is a glycoprotein hormone acting as the main regulator of erythropoiesis. Its physiological role becomes crucial when the delivery of oxygen to tissues/organs is compromised leading to low arterial O_2 partial pressure (pO_2) and, thus, low blood O_2 content, inducing Epo to stimulate the red blood cell (RBC) production in order to restore the blood O_2 carrying capacity [1]. The relationship between the O_2 content of the blood and the red blood cell synthesis has been described for the first time by the French anatomist François-Gilbert Viault. In 1890, during a 23 days trip to the highlands of Peru (Marococha, 4500 m), Viault measured an increase of his RBC from 5 to 8 million per µl blood, indicating that at high altitude the rarefied air, containing a low number of O_2 molecules per air volume, stimulated an adaptive response of the human body to increase the RBC number as well as the blood O_2 carrying capacity meeting the metabolic demand of the organism [2]. In 1906, Paul Carnot and Claude Deflandre of Paris proposed for the first time a hormonal regulation of erythropoiesis after observing that the injection of serum (5-9 mL) from anaemic donor rabbits into healthy recipient rabbits stimulated the production of the recipient's RBC by 20-40% within 1-2 days [3]. This reaction suggested the existence of a hormone, first called "hemopoietine", present in the donor's serum, which triggers the production of new erythrocytes. In 1948, Eva Bonsdorff and Eeva Jalavisto from Helsinki coined the specific term "erythropoietin" (Epo) [3]. However, the prove for an oxygen-dependent humoral factor regulating the RBC count came only later as attempts to validate the experiments of Carnot and Deflandre were unsuccessful. In 1953, Erslev administered large quantities of plasma (17 mL/kg, 50-200 mL, once a day for four days) from anaemic rabbits to normal rabbits resulting in the stimulation of erythropoiesis [4]. This convincing experiment demonstrated that the erythropoietic factor could pass through the anaemic to the normal blood and raise the amount of RBC. Indirect evidence supporting the hypothesis of Epo as an erythropoiesis regulator was provided by experiments of Reissmann, who in 1950 reported augmented RBC production in parabiotic rat-pairs where the circulation systems of both animals were connected by flaps of skin with one rat exposed to low oxygen tension (hypoxic condition) and the other to normal oxygen tension (normoxic condition). Reissmann explained this result by either the presence of erythropoietin in the blood of the rats or its transfer from the hypoxic to the normoxic rat [5]. Successively, Jacobson and Nathan, in 1957 and 1964, respectively, performed organ ablation experiments, in rats and humans, respectively, and concluded that the kidney is the major but not the exclusive site of Epo production [6] [7]. These findings encouraged Goldwasser and his colleagues to purify Epo from kidneys, which

turned out to be an unsuccessful strategy due to the release of proteolytic enzymes after tissue homogenization [8]. Therefore, his next steps to isolate Epo relied on the plasma of anaemic sheep and on urine from Argentinians with severe iron deficiency, but again these attempts revealed to be a fail, although important chemical properties about Epo as a protein were characterized. Importantly, on the Christmas day of 1975, Dr. Miyake brought to Chicago a package, almost like a Christmas gift, containing 2550 L of urine from Japanese patients suffering from aplastic anaemia in Kumamato City. Together with Goldwasser and colleagues, they finally managed to purify 8 mg of human Epo [9]. From that moment, the study of Epo took off and thanks to the determination of the amino acid sequence and to the recombinant DNA technology, two independent groups cloned the human *EPO* gene in 1985 [10] [11]. It became clear that a new era for Epo just started: pure Epo could help to broaden the knowledge about its function, regulation and therapeutical benefit. Soon, several clinical trials demonstrated the effectiveness of recombinant human Epo (rhEpo) for the treatment of anaemia related to chronic kidney disease. Eschbach et al. showed that, after receiving intravenous injections of rhEpo (doses between 15 and 500 units per kilogram of body weight) three times weekly after dialysis, the haematocrit of 11 out of 18 anaemic patients with end-stage renal disease (Hct= 20 ± 2) raised to 35% and no organ dysfunction or toxic side effects were reported, indicating that rhEpo is a valid theraupetical agent, which can abolish the requirement for transfusions and correct anaemia, improving the quality of life of thousands of chronic renal disease patients [12] [13]. Because Epo is a complex glycoprotein having 40% of its total molecular weight glycosylated, rhEpo has been mainly expressed in Chinese hamster ovary (CHO) cells for the large-scale pharmaceutical manufacture as mammalian cells ensure post-translational modifications including glycosylation. rhEpos deriving from CHO cells named epoetin-α (e.g. Epogen®) or epoetin-β (e.g. Epogin®) belong to the first-generation recombinant Epo or erythropoiesis stimulating agents (ESA) and have been used for 20 years in clinics. While the amino acid sequence is identical to endogenous Epo, such epoetins display a different glycosylation pattern despite being produced in the same mammalian cell host and having the same clinical efficacy. In fact, epoetin-α is more homogenous, showing less basic isoforms than epoetin-β. The reasons for such glycosylation differences can be attributed to the variability of the host cell, the transfected plasmids, the cell culture conditions and the downstream purification process [14]. As soon as the patent for these drugs expired, many more biosimilar recombinant Epo products entered the market, tagged as second-generation ESAs. These new biomolecules have a different glycosylation pattern and improved pharmacokinetic properties. For example, darbepoetin-α (e.g. Aranesp®), was obtained through modification of the amino acid sequence by site-directed mutagenesis, resulting in hyperglycosylated Epo, having an increased molecular weight but a three to four times longer half-life in the circulatory system

that allowed reducing the frequency of drug administration. rhEpo was further improved by chemical modification, leading to the PEGylated Epo or CERA, ‘’continuous erythropoiesis receptor activator’’, with a longer plasma half-life than darbepoetin-α [15]. Surprisingly, in 1996, by random phage display peptide libraries, Nicholas et al., identified small peptidic molecules with no structural analogy to Epo, capable of binding the extracellular domain of Epo receptor (EpoR) and of triggering its activation and an erythropoietic response in a cell-based context as well as *in vivo.* The discovery of these small Epo mimetics opened the possibility for a low cost therapy against anaemia in a long run, although further investigations about their mechanism of actions are required since serious adverse events were reported [16]. Remarkably, nowadays, rhEpo can be considered the most successful therapeutical application of recombinant DNA technology, reaching the largest market of biopharmaceuticals with annual sales rates of $\sim 10^{10}$ Euro [15].

Because recombinant Epo stimulates the production of new RBC increasing the blood oxygen carrying capacity and the amount of oxygen delivered to the muscles, it is not hard to imagine how fine the borderline between Epo use and Epo abuse is. In fact, rhEpo has been illegally adopted as a performance-enhancing drug and thus in 1990 it was banned by the International Olympic Committee (IOC) Medical Commission due to its potent doping function in endurance sports where an intense aerobic effort is required like cycling and cross-country skiing. Epo abuse became a scandal worldwide and the hormone Epo well-known also to the non-scientific community. Famous athletes admitted to have misused rhEpo to enhance artificially their performance during sport competitions, like the American cyclist Lance Armstrong who revealed their systematic doping with Epo for years. The detection of Epo in sport has been challenging due to multiple reasons: difficulty to differentiate between endogenous Epo and recombinant Epo; short plasma half-life (8.5 ± 2.4 hours when administered intravenously and 19.4 ± 10.7 hours when administered sub-cutaneously; undetectable in urine after 3-4 days of injection) [17] [18]. However, direct and indirect diagnostic tests have successfully been developed to measure Epo in endurance athletes. The direct test allow for the detection of Epo in urine by exploiting the different biochemical properties due to the different glycosylation pattern of endogenous and recombinant Epo that confers distinct net electrical charges and thus isoelectric points. Therefore, the endogenous and synthetic Epo can be distinguished by electrophoretic separation according to the isoelectric point. The indirect test, instead, rely on the determination of parameters that indirectly confirm the presence of Epo in the bloodstream like the haematocrit, reticulocytosis and macrocytosis, which reflect increased bone marrow activity. Additionally, the assessment of the soluble form of the transferrin receptor (sTfR) is useful to diagnose not only the erythropoietic activity in the bone marrow but also the iron status in the body [19]. sTfR levels

are increased when erythropoiesis is stimulated and in conditions of iron deficiency occurring during anemia, or when the iron demand is elevated as in the case of stimulated erythropoiesis. On the other hand, sTfR values are decreased when the iron levels are augumented, a condition that can be found during doping as athletes often accompany the rhEpo injections with iron supplementation in order to supply the newly formed RBC with adequate amounts of iron [20]. Of note, injections of rhEpo can have dangerous side effects as the formation of new RBC induced by Epo in the bone marrow renders the blood viscous, thus, resulting in a high risk of thrombosis. Indeed, the enhanced haematocrit (above 50%) and the blood viscosity can provoke heart failure, a fatal event that caused the suspicious death of some athletes during sleep when the heart rate is slower [21]. An increase of the haematocrit does not correlate linearly with improved endurance as shown by transgenic mice carrying additional copies of the *Epo* gene having 90% haematocrit but low aerobic performance [22]. What value of haematocrit gives then the maximal oxygen carrying capacity? Exercising wild-type mice administered with an ESA (acute model) and transgenic mice overexpressing Epo (chronic model) were shown to reach the highest maximal oxygen uptake at haematocrit levels of 58% and 68%, respectively [22]. Surprisingly and in agreement with this study, the Finnish cross-country skier Eero Mantyranta, winner of two gold medals at the 1964 Winter Olympic Game, was identified to carry a mutation in the Epo receptor (EpoR) gene that caused prolonged activation of EpoR and of erythropoiesis keeping low the plasma Epo levels. Mantyranta's blood oxygen carrying capacity was increased by 25–50% and his haematocrit was of about 68%, close to the optimal value for improved sport performance as verified by *in vivo* studies [22, 23]. Such genetic mutation conferred him a great advantage, being considered as "natural doping" [18].

Epo plays clearly an essential role in sustaining the adaptive process of the human body to conditions of reduced oxygen availability by enhancing the number of RBC when the oxygen supply is limited. However, it is important to keep in mind that Epo is not only a therapeutic agent, which saved the life of milions of anaemic patients, but also that an excessive use of recombinant Epo causes increased blood viscosity, eventually leading to heart failure and death. Therefore, it is necessary to keep the Epo levels within a specific range. The actual dose of rhEpo recommended in the therapy against anaemia varies between individual patients, depending on their specific clinical condition but remains in the range of 75 to 120 IU/kg per week including iron supplementation to maintain haemoglobin (Hb) levels of 11 to 13 g/dl [21].

Although Epo therapy is very efficient, it remains still expensive as anaemic patients affected by kidney diseases need lifelong rhEpo injections. Therefore, the study of Epo regulation in physiology is of particular interest as it will help to decipher the molecular players involved

into Epo dysregulation during renal pathologies. The knowledge of the genetic elements as well as the proteins contributing to modulate Epo expression will open the possibility in the near future to design new drug targets and develop a new Epo therapy based on smaller and cheaper molecules [21].

1.2 Physiological role of Epo

Epo is the master regulator of red blood cell homeostasis. By stimulating erythropoiesis, Epo maintains the balance between the ~120 days old removed RBC and the newly produced RBC. In presence of reduced oxygen tension in the tissue (hypoxia), decreased content of haemoglobin or reduced number of RBC (anaemia), Epo production is stimulated in the adult kidney and the fetal liver whereas the adult liver contributes only about 15% to Epo production [24] [25]. Newly produced Epo is then secreted into the bloodstream, reaches the bone marrow where it binds its specific receptor (EpoR) and activates erythropoiesis, restoring the O_2-carrying capacity of the blood to physiological levels (Figure 1) [8] [21] [1].

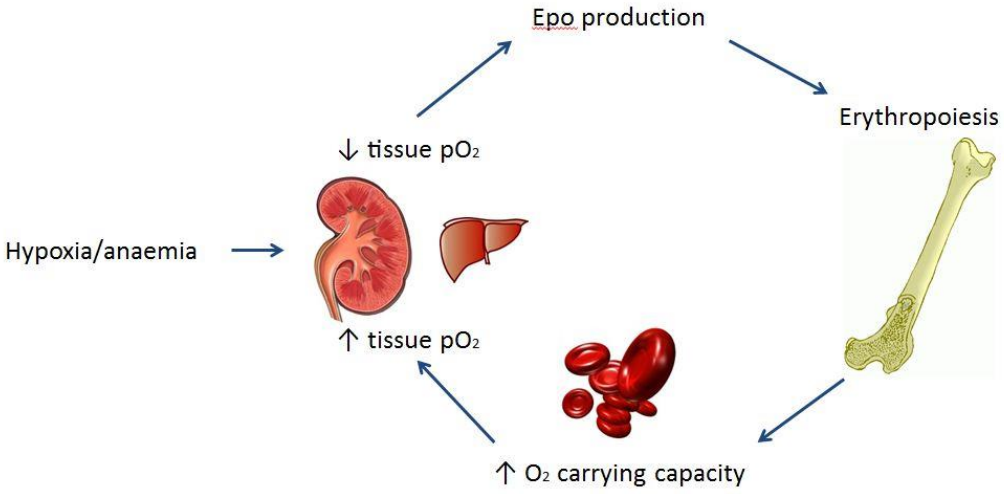

Figure 1. Epo regulates red blood cell formation upon tissue hypoxia or anaemia.

1.2.1 Erythropoietic function

The primary function of Epo is the regulation of erythropoiesis. Erythropoiesis occurs in the fetal liver during development and in the bone marrow at adulthood. Particularly, Epo must ensure that the RBC mass and the haemoglobin concentration remain constant day by day and that in case of haemorrhage, the recovery of the RBC count is fast [1]. The *modus operandi* of Epo consists in supporting the survival, proliferation and differentiation of erythroid progenitor cells in the bone marrow. The EpoR is located on the surface of the colony-forming unit erythroid (CFU-Es) and EpoR activation induces their differentiation to

mature erythroblast. Erythropoiesis is a slow-acting process, where the amount of new erythroblasts becomes significant only after 3-4 days following an increase in plasma Epo [1]. The erythropoietic action of Epo can also be influenced by hormonal factors: erythropoiesis is further stimulated by androgens, increasing haemoglobin concentration and red blood cell counts in men, while in women erythropoiesis is less stimulated by oestrogens, leading to decreased values of haematocrit and haemoglobin concentration compared to men [1]. Interestingly, notwithstanding the sex differences in haematocrit and haemoglobin concentration, the plasma Epo levels between women and men remain similar, indicating that the lower Hct and Hb values of women belong to a physiological range [26]. As shown in Figure 2, erythropoiesis starts in the adult bone marrow from the pluripotent hematopoietic stem cells (HSC), which differentiate into immature erythroid progenitor burst-forming unit erythroid (BFU-E) cells that in turn differentiate into CFU erythroid (CFU-E) cells. Erythroid progenitors (BFU-E and CFU-E) are capable to form colonies of maturing erythroid cells *in vitro*. Morphologically, erythroid precursors are distinguished as proerythroblasts (Pro EB), basophilic erythroblasts (baso EB), polychromatophilic erythroblasts (Poly EB), and orthochromatic erythroblasts (Ortho EB). The maturation of CFU erythroid precursor to proerythroblast is Epo-dependent as shown by the presence of EpoR that sensitises the CFU-E cells to Epo. The differentiation into erythrocytes elicited by Epo causes three major effects: Epo acts as anti-apoptotic factor, thus promoting the erythroid progenitor survival; Epo stimulates cellular expansion and cell division by inducing RNA polymerase activity and DNA synthesis; Epo enhances the production of transferrin receptor, integral membrane proteins and haemoglobin, as highlighted in Figure 2 by the red baso EB stage.

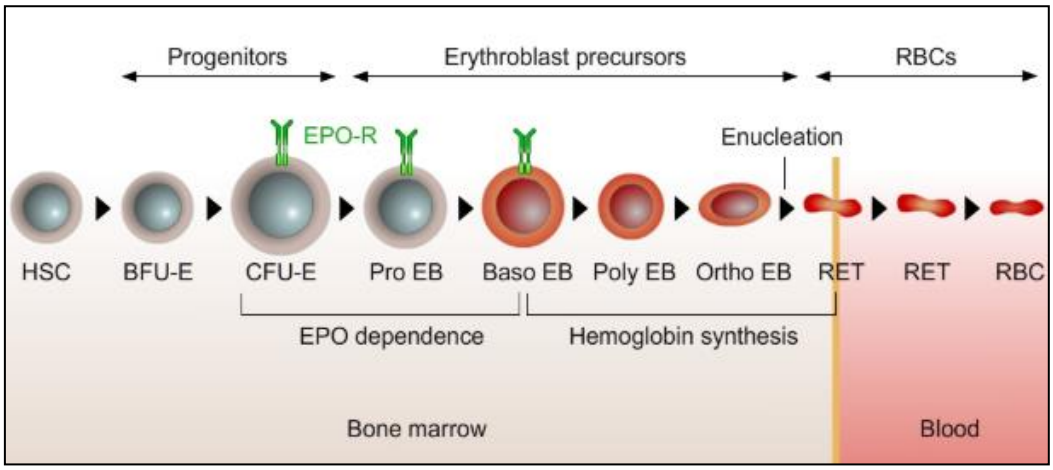

Figure 2. Stimulation of erythropoiesis through Epo. From [27].

Downstream the proerythroblast stage the differentiation steps are independent from Epo. The erythrocyte maturation takes place within erythroblastic islands, composed of erythroblasts physically attached to central macrophage cells and consist in enucleation of

the ortho EB to form two cell populations: reticulocytes (RET), which are irregularly shaped cells containing haemoglobin and residual organelles (the reticulum) [28] and pyrenocytes ("extruded nuclei") containing condensed nucleus surrounded by a lipid bilayer and thin rim of cytoplasm [29].

The erythropoiesis process described so far, which takes place in the late stage of fetal life in the liver and in the postnatal bone marrow is called "definite erythropoiesis", to distinguish it from the "primitive erythropoiesis", occurring in the early embryonic developmental stage. In fact, the first blood cells of the mammalian embryos originate from "blood islands" of the yolk sac. Studies in mouse embryos highlighted the role of a unique erythroid progenitor termed Egypt-CFC emerging from the yolk sac at E7.25, peaking at E8.25 and undetectable at E9.0 [30]. *In vitro,* Egypt-CFC like their definitive erythroid progenitors requires Epo for developing the colonies of maturing erythroid cells. The primitive erythroid cells undergo progressive morphological maturation similar to their definitive counterparts between E9.5 and E12.5: symmetric cell division, accumulation of haemoglobin, reduction of cell size, nuclear piknosis and decrease in RNA content.

The switch in the expression between embryonic, fetal and adult globin genes depends on the activation of specific transcription factors which regulate the expression of adult globin genes but not embryonic globin gene [31]. Indeed, the transcription regulators Sox6 and Bcl11A have been found exclusively in definitive erythroid cells, where they suppress the expression of embryonic globin genes [32]. Importantly, by employing specific antibodies against embryonic mouse globin, it has been discovered that primitive mouse erythroblast cells lose their nuclei between E12.5-E16.5 of gestation [33], confuting the misconception that primitive erythroblast remain nucleated for the whole lifespan in the fetal circulation.

Epo in primitive and definitive erythropoiesis

The role of Epo in primitive erythropoiesis has been debated for a long time. While previously small amounts of Epo have been detected in the early mouse yolk sac [34], EpoR protein has been quantified on the surface of maturing primitive erythroblast in the fetal hamster [35] and EpoR transcripts were found in yolk sac blood islands between E7.5–E8.5 and in the yolk sac of E9.6–11.5 mouse embryos [36]. In contrast to previous *in vivo* studies [37], recent studies highlighted the involvement of EpoR signaling through primitive erythropoiesis as EpoR-null mouse embryos showed reduction of cell proliferation and increased apoptosis [34]. New findings elucidating the role of Epo in primitive erythropoiesis come from the study by Suzuki et al., who demonstrated that Epo is required for the maturation of the embryonic erythroid cells since Epo knock-out mouse embryos (Epo-KO) were moderately and severely anaemic at E10.5 and E11.5, respectively, and died due to anaemia at E12.5, in agreement

with previous observations [37] [38]. Surprisingly, by dissecting these mouse embryos, the group of Yamamoto was able to detect Epo mRNA levels exclusively in the head region of E8.5 embryos appearing as the first Epo producing tissue during mouse embryogenesis as well as in the head and fetal liver of E9.5 and E10.5 embryos (Figure 3) [39]. Additionally, they reported that Epo production switches from the head region to fetal liver at E11.5 during mouse development. To define the specific cellular type able to produce Epo during embryonic mouse development, Suzuki et al. employed transgenic mouse lines carrying GFP reporter transgenes encompassing different length fragments within the 5' upstream region of the *Epo* gene. They could show that cells of the neuronal tissue, a subpopulation of neural and neural crest cells, exhibited reporter green fluorescence indicating that they were capable to express Epo. Therefore, Epo appears to support erythropoiesis already during mouse development [39]. The importance of Epo as main regulator of definitive erythropoiesis has been proved through Epo or EpoR knock-out animal studies. Both $Epo^{-/-}$ and $EpoR^{-/-}$ mice were reported to have yolk sac erythropoiesis heavily decreased and impaired definitive erythropoiesis, which leads to embryonic lethality by E13-E15 due to severe anemia [37], demonstrating a crucial role for Epo and EpoR in erythropoiesis. Additionally, in fetal livers derived from $Epo^{-/-}$ and $EpoR^{-/-}$ mice both BFU-E and CFU-E progenitors were found, showing that the deficiency in erythropoiesis does not occur at erythroid progenitor stage and thus the major function of Epo is to trigger proliferation and definitive differentiation of CFU-E but not of earlier progenitors [40].

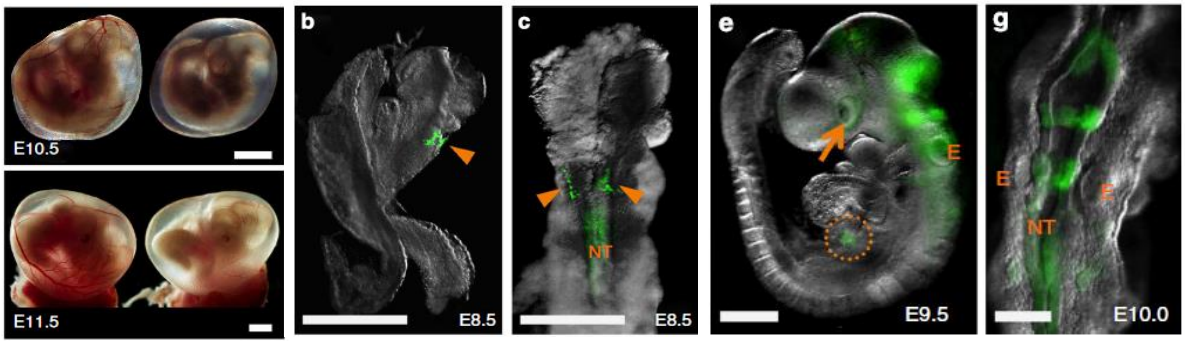

Figure 3. Embryonic neuronal tissues transiently express Epo during mouse gestation. Left panel: Wild-type (left) and Epo-KO embryos (right) at the indicated stages (E10.5 and E11.5). Scale bars, 1.0 mm. Right panel: Transgenic mouse embryos at E8.5 (b,c), E9.5 (e) and E10.0 (g). GFP-positive cells are observed in or around the neural folds (orange arrowheads) at E8.5; the fetal liver (dotted circle) at E9.5; in optic vesicle (orange arrow) and otic vesicle (orange E) at E9.5; in otic vesicle (orange E) and neural tube at E10.0 (orange NT). Scale bars, 0.5 mm (b,e) and 0.2 mm (c,g). Adapted from [39].

In fact, Epo prevents apoptosis of the CFU-E progenitors by acting as prosurvival signal upon EpoR binding at the CFU-E surface, as verified by *in vitro* experiments where in the absence of Epo CFU-E progenitors undergo apoptosis [41].

Plasma Epo concentration

Several transgenic mouse models overexpressing Epo were shown to be affected by erythrocytosis having increased number of RBC, hematocrit and haemoglobin levels [42] [43] [44]. These results highlight the aspect that erythropoiesis is dependent on Epo levels and, therefore, it is necessary to maintain Epo concentration in the physiological range to avoid either excessive erythropoiesis, leading to polycythemia, or heavily reduced erythropoiesis, causing anemia. How much Epo do we have in our body at physiological condition? Plasma Epo concentration varies between 6 and 10 mU/mL in healthy humans, independent of gender, with maximum peak at around midnight and minimum peak at around noon [45] [46]. However, the absolute concentration of Epo in the blood is not a sufficient parameter to verify whether Epo production in our body is adequate, but rather the Epo production in relation to the oxygen availability indicated by the haemoglobin concentration is a more reliable parameter to estimate the formation of Epo [21]. Indeed, an inverse semilogarithmic relationship between plasma Epo concentration and haemoglobin concentration or hematocrit exists (Figure 4), which has been discovered through studies in humans and mice, and is independent of the method employed to induce the reduction in haemoglobin concentration, i.e. irradiation, bleeding or hemolysis [47] [21].

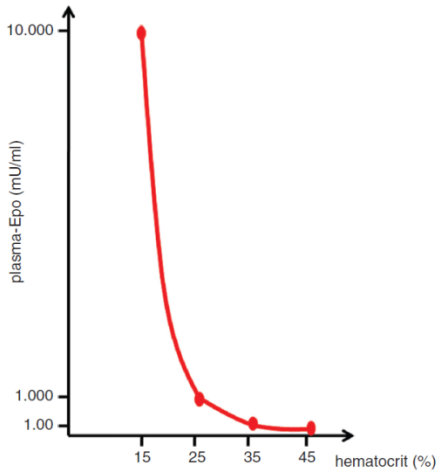

Figure 4. Inverse semilogarithmic relationship between plasma Epo concentration and hematocrit in anaemic rat. From [47].

Upon hypoxic or anemic stimuli Epo plasma concentration can increase up to 1000-fold (10 nmol/liter), depending on the degree of severity during anemia or inspiratory hypoxia [21]. However, human studies demonstrated that moderately raised levels of Epo such as 100 pmol/liter are sufficient to trigger erythropoiesis; therefore, it remains unclear why the plasma Epo concentration is so highly increased in anemia or arterial hypoxia. Surprisingly, in case of complex form of anemia, such as anemia due to chronic inflammation or cancer, the

typical inverse relationship between Epo plasma concentration and hematocrit/haemoglobin is not anymore valid as it flattens, indicating that Epo production is inappropriately decreased in those patients and the reason why is still unknown [48] [49].

Stimuli for Epo production

The stimulus for Epo production is not the decrease in arterial oxygen tension but the reduction of oxygen partial pressure in Epo-producing tissues, occurring when the Hb O_2 saturation is decreased (hypoxemia) or the oxygen-carrying capacity of the blood is reduced (anemic hypoxia). Hypoxemia can be caused by a decline in ambient tension of oxygen (e.g. high altitude), by pulmonary dysfunction (e.g. pulmonary edema, hypoventilation) or by heart disease (e.g. right to left shunt). Anemia can be caused by blood loss (e.g. hemorrhage), defective erythropoiesis or hemolysis due to infections like malaria, or as a consequence of a hemoglobinopathy, a disease characterized by red blood cells having scarce functional haemoglobin. Moreover, the oxygen affinity of haemoglobin plays a pivotal role in regulating the oxygen release to the tissues. In fact, when the oxygen affinity of haemoglobin is elevated, the O_2 binding increases, the degree of O_2 saturation raises, impairing the oxygen release to the tissues and elevating Epo levels. On the contrary, a reduced oxygen affinity of haemoglobin favors O_2 release and improves tissue oxygenation [50] [21].

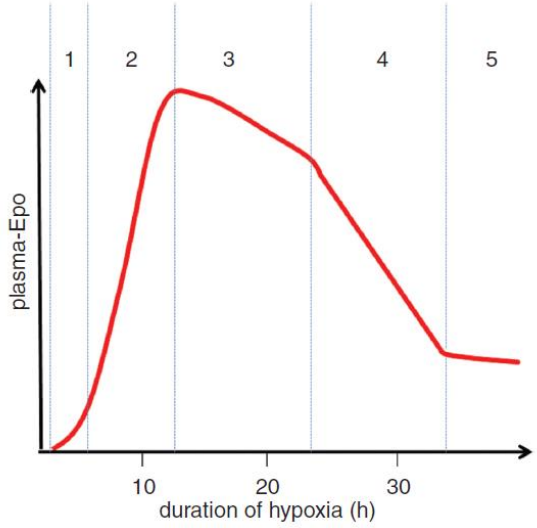

Figure 5. Plasma Epo kinetics during acute inspiratory hypoxia. Time course of plasma Epo concentrations in rats exposed to inspiratory hypoxia (60 mmHg) in a normobaric atmosphere. Epo levels start increasing after a delay (phase 1), then Epo levels increase linearly (phase 2) reaching a peak (phase 3) and declining (phase 4). Finally, Epo levels reach a steady-state level (phase 5). From [21].

The kinetics of Epo response to a decreased oxygen tension (Figure 5) has been deeply analyzed: following a drop of O_2 tension in the alveoli, and consequently in arterial plasma, Epo levels begin to increase after 60 to 90 min from the onset of the hypoxic stimulus and continue to increase linearly with a slope that depends on the severity of hypoxia and

reaches a peak between 12 and 24 h in rodents and within 48 h in humans [51]. After that, Epo diminishes to a steady-state level that is lower than the peak but higher than the baseline with Epo levels inversely related to the severity of the inspiratory hypoxia [21]. Interestingly, the decline of plasma Epo concentration is correlated with a decrease in Epo mRNA levels and takes place before the increase of RBCs. By Northern blot analysis, Eckardt et al. showed that the early reduction in Epo production is not caused by a negative feedback regulation through Epo itself but rather due to a decrease in renal Epo mRNA content, which the author assumed to be caused by reduced Epo mRNA stability [52]. Subsequent studies focused on Epo mRNA stability reported a 50-fold increase of Epo mRNA levels in response to hypoxia or $CoCl_2$ by Northern blot analysis and an increase of Epo gene transcription of only 10-fold by nuclear run-off experiments, indicating that both the rate of gene transcription and post-translational events regulate Epo mRNA levels [53]. Later, Rondon et al. found that the erythropoietin mRNA-binding protein (ERBP) binds to pyrimidine-rich 120-bp region in the 3' UTR of Epo mRNA and that the ERBP complex formation was increased under hypoxia conditions [54]. A follow-up study from the same group demonstrated that Epo mRNA stability is enhanced due to the binding of ERBP to the 3' UTR of Epo mRNA and confirmed that the increase of Epo mRNA stability in hypoxia is accompanied by an increase in ERBP complex synthesis [55].

Finally, the Epo response to hypoxia is threshold-dependent: reduction of arterial pO_2 to a certain threshold (about half of the normal arterial pO_2 value) has only a small influence on circulating Epo levels whereas a further decrease of oxygen tension below this threshold induces an exponential increase of plasma Epo levels in humans and rodents [56] [52].

1.2.2 Non-erythropoietic function of Epo

Epo exerts non-hematopoietic effects beyond its classical erythropoietic function. In fact, Epo mRNA together with EpoR mRNA and/or EpoR protein expression have been reported in non-hematopoietic tissues (Figure 6), including megakaryocytes [57], endothelial cells [58] [59], brain [60], placenta [61], testis [62], vascular smooth muscle cells [63], and in several cancer tissues. Since the endogenous levels of Epo detected in all these organs/tissues are lower than in the kidney, it has been hypothesized that such small amounts of Epo act locally following paracrine/autocrine mechanisms. However, the physiological relevance of such observations is still a matter of debate. Sinclar and collegues, for example, were able to detect only very low levels of EpoR transcripts in extra-hematopoietic Epo-producing human endothelial, cardiac, neuronal and renal cells. As the authors could not detect the EpoR protein on the cellular surface, at that time they suggested the lack of specificity of previous commercial EpoR antibodies as the cause of undetectable signal. Additionally, Elliott et al.

reported failure of EpoR downstream signalling activation after *in vitro* administration of high doses of recombinant Epo in non-hematopoietic tissues [64] [65]. Endothelial cells from caesarean section-derived HUVECs are the first non-hematopoietic cell type to be reported to express EpoR, to bind Epo and to have functional Epo-EpoR axis, leading to a proliferative response, angiogenesis [66] and protection from apoptosis [67] [68]. Endothelial cells are known to express endothelial nitric oxide (NO) synthase (eNOS), which synthetize NO to regulate vascular tone and blood flow. *In vitro* studies performed under hypoxic conditions combined with recombinant Epo treatment showed increased EpoR mRNA and protein levels, activation of EpoR signalling pathway through enhanced AKT1 (Rac-alpha serine/threonine protein kinase) as well as stimulation of NO production, while in normoxic conditions no Epo mediated AKT1 activation was detected and only few transcripts of EpoR were expressed [69].

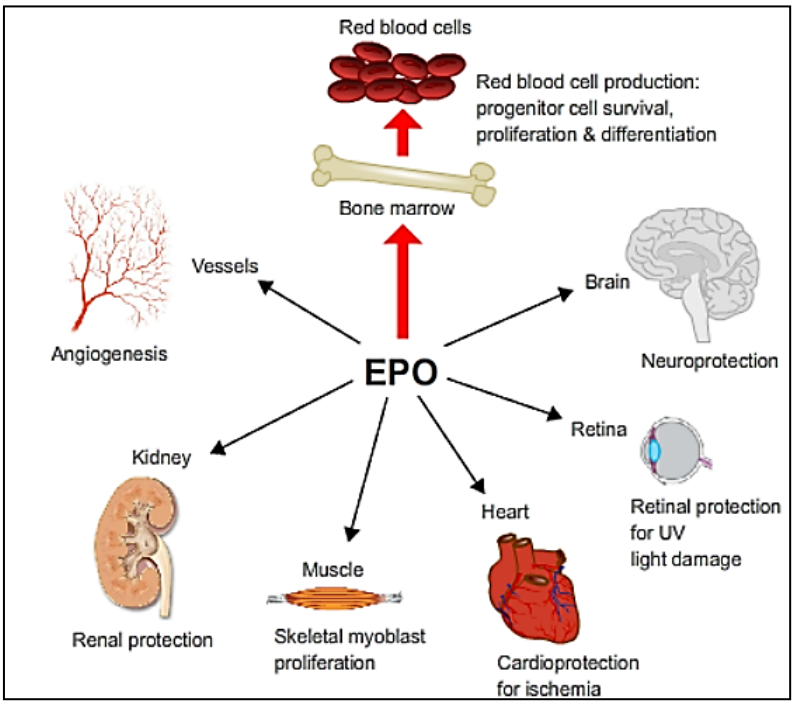

Figure 6. Non-erythropoietic effects of Epo. Epo is a cytoprotective agent acting in an autocrine/paracrine manner on several non-hematopoietic tissues, including the brain, the heart, blood vessels and the kidneys. From [70].

Interestingly, transgenic mice overexpressing Epo (*tg6*) and having high haematocrit did not develop hypertension as the increased levels of circulatory eNOS and NO ensured appropriate modulation of the vascular tone. In line with these *in vitro* experiments, recombinant Epo administration in rodents also stimulated NO production [71] [72] [73]. The increase in NO amounts might be considered as an acute response to facilitate the oxygen delivery through the regulation of the vascular tone occurring before the production of new

red blood cells upon hypoxia is completed [74]. It has been also shown that Epo can stimulate angiogenesis after tissue injury by acting synergistically with VEGF (Vascular Endothelial Growth Factor), promoting vascular integrity of the endothelial barrier [75]. The involvement of Epo/EpoR axis in angiogenesis has been also confirmed by $EpoR^{-/-}$ mice, which exhibited severe vascular defects as well as reduced complexity of the vessel network already at E10.5, 3 days before the embryonic lethality [76].

The anti-apoptotic and proliferative effects of the Epo/EpoR signalling as well as the enhanced NO production have been observed also in brain [77] and in heart [78]. The heart is prone to be affected by hypoxia as in infarction and atherosclerosis. Of note, EpoR has been detected on cardiomyocytes and during development $EpoR^{-/-}$ mice exhibit ventricular hyperplasia due to inadequate cell proliferation and myocardium expansion [38]. In animal models of heart infarction and ischemia-reperfusion injury, Epo treatment reduced the cardiac damage showing a cardio protective effect [79] [80]. Fiordaliso et al. employed a carbomylated form of recombinant Epo with no erythropoietic effect that did not increase the haematocrit while protecting the myocardium [81]. The proposed mechanism behind the cardio protection assumes the opening of potassium channels and the activation of signalling pathways downstream EpoR such as JAK/STAT, P13K/akt, and p38 MAPK, that inhibit apoptosis, stimulating proliferation [82]. The involvement of EpoR has been proven through $EpoR^{-/-}$ mice rescued with an erythroid specific EpoR transgene that showed less STAT3 phosphorylation due to lack of functional EpoR in cardiomyocytes. Furthermore, while the reduction of cardiac ischemia/reperfusion injury mediated by Epo treatment induces NO production by increased eNOS activity in cardiomyocytes [83], EpoR expression restricted to hematopoietic and endothelial cells showed a similar increase in eNOS activity in coronary artery endothelium and myocardial ischemia protection as in wild-type animals [78], indicating that the endothelial cell response to Epo is sufficient to trigger an acute cardio protection.

As mentioned above, EpoR expression has been also found in cancer tissues, suggesting the possibility to treat anaemia associated with chemotherapy with recombinant Epo, promoting the increase of haemoglobin and haematocrit. On the other hand, several clinical studies reported increased thrombosis [84] and high risk of tumour growth via the activation of Epo/EpoR signalling causing stimulation of cancer cell proliferation in addition to enhanced tumour oxygenation due to the erythropoietic effect of Epo treatment [85] [86] [87].

Recently, the existence of two different pathways responsible for the erythropoietic and non-erythropoietic functions of Epo has been proposed since a new potential isoform of EpoR was identified. While hypoxic circulating renal Epo binds in the picomolar range high-affinity

EpoR in bone marrow, inducing its homodimerization to activate proliferation and differentiation to mature red blood cells, locally paracrine and autocrine Epo produced in healthy tissue that surrounds the tissue damage, binds in the nanomolar range an isoform of low-affinity EpoR, which heterodimerizes and activates anti-apoptotic and pro-survival signalling cascades with cytoprotective effects that protect the tissue from further injury [88] [89].

Surely, the discovery of non-erythropoietic effects of Epo opens an interesting area of research as well as the possibility of new therapies based on modified and non-erythropoietic versions of recombinant Epo (e.g. carbomylated Epo) which avoid potential erythropoietic side-effects but support the protection of the non-erythropoietic tissue during ischemia-reperfusion, trauma, cytotoxicity or inflammation [90]. In this regard, Brines et al. demonstrated that rhEpo can reach the central nervous system (CNS) and accumulate in the cerebrospinal fluid (CSF) in a time and dose dependent-manner, opening the possibility to apply intravenous administration of rhEpo to patients affected by brain disorders. In contrast to previous studies, claiming that Epo could not cross the blood brain barrier (BBB) due to its impermeability to highly glycosylated molecules, the authors suggested that Epo could cross the BBB by transcytosis upon binding of EpoR molecules,which were found to be localised around brain capillaries within astrocytic endfeet and on the luminal side of capillary endothelial cells. Since the cerebral ischemia is associated with an opening of the BBB, the intravenous application of Epo could be a promising treatment of brain disorders [91].

1.3. Structure of Epo and Epo Receptor

Structure of erythropoietin

Erythropoietin is a glycoprotein hormone of 165 amino acids, synthesized as a precursor protein containing a signal peptide (residue 1-27), removed, during protein maturation. The structural architecture of Epo consists in an up-up-down-down four-α-helical bundle topology (Figure 7) αA (residue 8-26); αB (residue 55-83); αC (residue 90-112); αD (residue 138-161)) with two mini-α-helices located between the carboxy-terminus of the AB loop and the helix C as well as two short antiparallel β-strands in positions 39-41 and 133-135, respectively. The whole structure is stabilized by two disulphide bridges between Cys7 and Cys161, connecting the long antiparallel helices αA and αD, whereas the other antiparallel helices αB and αC are connected by a short loop. The long AB loop is required for the interaction between Epo and EpoR and it is linked to the αA through the second disulphide bond, Cys29- Cys33. The hydrophobic core of Epo is composed by non-polar amino acids of the αA, αB, αC helices, bound to the hydrophobic amino acids Phe138, Phe142, Tyr145,

Phe148, Leu153, and Tyr156 located at the internal surface of the αD helix [92] [93]. Erythropoietin is highly glycosylated as the carbohydrate portion amounts to more than 40% of the total molecular weight of 30.400 Da. The protein backbone comprises three N-glycosylation sites (Asn24, Asn38, and Asn83) and a single O-glycosylation site (Ser126). While the N-glycosylation sites are conserved in Chordata from fish to primates, suggesting a functional relevance of such post-translational modifications, the functional importance of the O-glycosylation site (Ser126) remains unknown [94]. In line with these observations, it has been reported that the therapeutic activity of Epo depends on the three aforementioned *N*-glycans as without them Epo has a very short-life, from 5 to 6 hours (average of endogenous human Epo half-life) [56] to few minutes, as well as almost complete loss of its functionality *in vitro* [95] and *in vivo* [96].

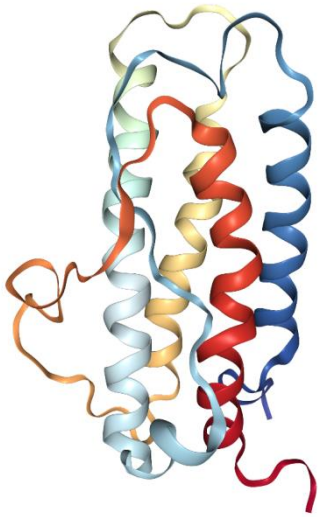

Figure 7. Three-dimensional structure of the human Epo molecule. 3-D structure of human erythropoietin obtained by nuclear magnetic resonance spectroscopy**.** Structural data were taken from the Protein Data Bank (http://www.rcsb.org/structure/1BUY; PDB ID: 1BUY; [97]) and displayed using the NGL Viewer [94].

Interestingly, Erbayraktar and colleagues demonstrated that a modified version of Epo molecule “asialoerythropoietin”, lacking completely the erythropoietic function, shows strong neuroprotection [98]. Overall, it appears evident that the carbohydrate structures have a pivotal role for the biological activity of erythropoietin. In fact, they are crucial for the binding of Epo with its receptor and the subsequent activation of the signalling cascade downstream [21]. The glycosylation of Epo is also important to maintain its survival while it circulates into the bloodstream as shown by increased clearance of deglycosylated Epo in liver *in vivo* [21].

The amino acid sequence of Epo exhibits high degree of interspecies similarity in mammals and the protein structure is highly conserved. Human Epo is 91% identical to monkey Epo, 85% to cat and dog Epo, and 80% to 82% to pig, sheep, mouse, and rat Epos. Full

conservation of the disulphide bridge linking Cys7 and Cys161 (NH2 and COOH termini) and of the N-glycosylation sites has been reported [99].

Although it has not been yet clarified whether Epo exists in lower organisms, the presence of Epo genes has been identified in teleost fishes such as fugu, zebra fish and trout [100] as well as in the amphibious Xenopous laevis [101].

Structure of Epo Receptor, EpoR

EpoR is encoded by a single copy gene with highly conserved sequence, encompassing eight exons. The erythroid Epo Receptor is a single-pass plasma membrane glycoprotein of 484 amino acids with a molecular weight between 72 and 78 kDa, which is mainly expressed as homodimer on the cellular surface of the erythroid progenitor CFU-E. The Epo-R belongs to the type I cytokine receptor superfamily and shares structural motifs with members of this receptor family, including two extracellular fibronectin type III-like domains, four similarly spaced cysteine residues forming two disulphide bonds in the extracellular region and a conserved Trp-Ser-X-Ser-Trp (WSXWS) motif in proximity to the transmembrane domain [102]. In addition to the extracellular region, EpoR contains a transmembrane segment as well as a cytosolic domain with no enzymatic functionality associated with the Janus tyrosine kinase 2 (JAK2) (Figure 8A). The activation of the EpoR signalling cascade takes place upon ligand binding, which induces dimerization and reorientation of EpoR monomers, bringing the two pre-associated JAK2 proteins close to each other and stimulating transactivation of JAK2 by phosphorylation [103]. As shown in Figure 8B, EpoR is a dimeric receptor, bound by a single Epo molecule in a 2:1 EpoR:Epo ratio [103].

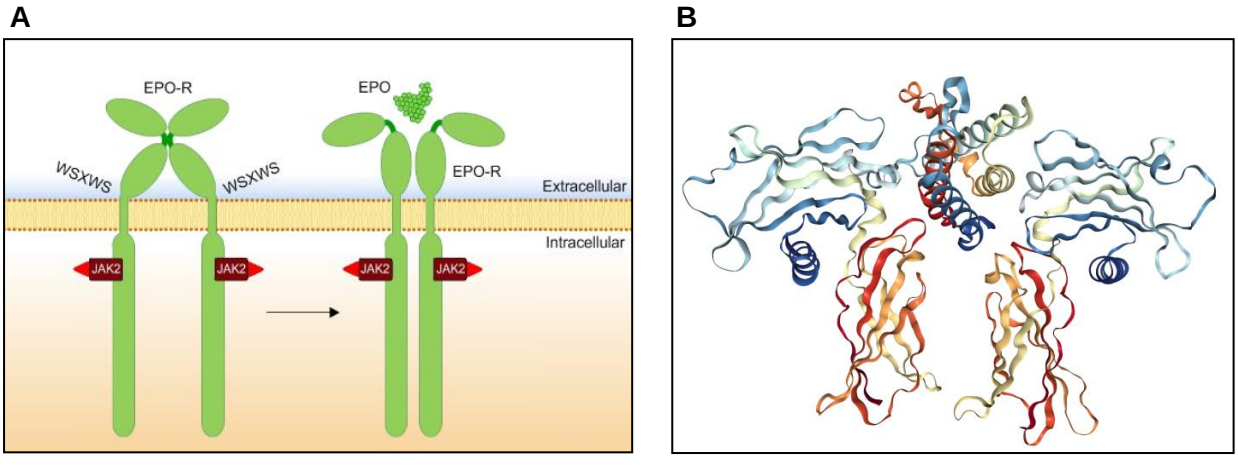

Figure 8. Structure of the human Epo receptor, EpoR. A) The EpoR comprises an extracellular region with circa 230 amino acids folding into two fibronectin III-like domains containing the WSXWS motif close to the membrane-spanning segment; one transmembrane segment and a cytosolic domain of ~ 230 amino acids with no enzymatic activity. The cytosolic proximal region is associated with the Janus tyrosine kinase 2 (JAK2) (red boxes). From https://kidneynccr.bio-med.ch. B) 3-D structure of human EpoR in complex with Epo obtained by X-RAY diffraction at 1.9 Å resolutions**.** Structural data

were taken from the Protein Data Bank (http://www.rcsb.org/structure/1EER PDB ID: 1EER; [103]) and displayed using the NGL Viewer [104].

Interestingly, EpoR dimer forms already during protein translation of the receptor and is then transported to the plasma membrane where it binds its ligand [105]. The EpoR-Epo complex revealed the existence of two Epo binding sites within EpoR: one high affinity binding site with a dissociation constant of approximately 1 nM, including a motif with a hydrophobic core surrounded by hydrophilic amino acids and a second binding site which interacts with Epo using a different set of residues, having a dissociation constant of 1 μM [106]. Taking into consideration both the high and the low affinity interactions between EpoR and its ligand, a sequential binding model for Epo-mediated activation of EpoR has been proposed, in which Epo binds first the high affinity site of one receptor chain and then the second lower affinity site within the second receptor chain [107]. The importance of receptor dimerization has been validated by mutating either Epo molecule in R103A or EpoR in the binding regions for Epo, resulting in the impairment of EpoR signalling cascade activation in hematopoietic cells [107] [108].

1.4 Epo Receptor signalling

As mentioned above, the signal transduction mediated by EpoR is initiated by ligand binding. Once Epo is bound, a conformational change with re-organization of EpoR monomers occurs, leading to transphosphorylation of the two cytosolic receptor associated Janus tyrosine kinase 2 (JAK2) proteins. As depicted in Figure 8A, JAK2 proteins are constitutively bound to the intracellular domains of EpoR and function as chaperone, assisting the transit of EpoR from the endoplasmic reticulum to the plasma membrane [109]. Notably, $Jak2^{-/-}$ animals die during embryogenesis at E12-E13 due to severe anaemia, similarly to $Epo^{-/-}$ and $EpoR^{-/-}$ mice, suggesting a pivotal role for JAK2 in Epo-dependent definitive erythropoiesis [110]. Phosphorylated and activated JAK2 kinases are able to phosphorylate tyrosine residues located in the membrane-distal portion of the cytoplasmic region of EpoR, which serve as docking sites for intracellular signalling molecules containing SRC homology 2 (SH2) domains such as STAT5, phosphatidyl-inositol 3-kinase (PI-3K) and the SH2 adaptor protein C (SHC) (Figure 9). As soon as these proteins are bound to EpoR, they become activated through phosphorylation and activate different signalling pathways, which include the signal transducer and activator of transcription (STAT) pathway, the phosphatidylinositol 3'-kinase/protein kinase B (PI-3K/PKB) pathway and the MAPK (mitogen-activated protein kinases) pathway [111].

As represented by Figure 9, in erythroid cells phosphorylated STAT5 dissociate from EpoR, homodimerizes and translocates into the nucleus, where it binds to its consensus site

TTN_5AA in the promoter of target genes [112] [113], activating the transcription of genes involved in survival, proliferation and differentiation of erythroid precursors, such as Bcl-xL. Bcl-xL is an anti-apoptotic protein, reported to inhibit apoptosis by modulating the activation of caspase proteases as well as by avoiding the release of cytochrome C from mitochondria through a mechanism that remains still unknown [114] [115]. The PI-3K/PKB pathway involves activation of the phosphatidylinositol 3'-kinase (PI-3K) and conversion of its catalytic domain of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Figure 9). Then, protein kinase B (PKB/Akt) binds PIP3 at the plasma membrane, becomes activated and translocates to the nucleus where it activates the transcription of pro-survival genes. Furthermore, the MAPK pathway comprises the binding of the adaptor protein SHC and growth factor receptor-bound protein 2 (GRB2) at phosphorylated tyrosine docking sites of EpoR (Figure 9), leading to the activation of RAS/RAF, MEK as well as the extracellular-regulated kinases 1/2 (ERK1/2), that in turn activate the transcription factor AP1 (activator protein 1) that regulates cellular differentiation, proliferation and apoptosis [74].

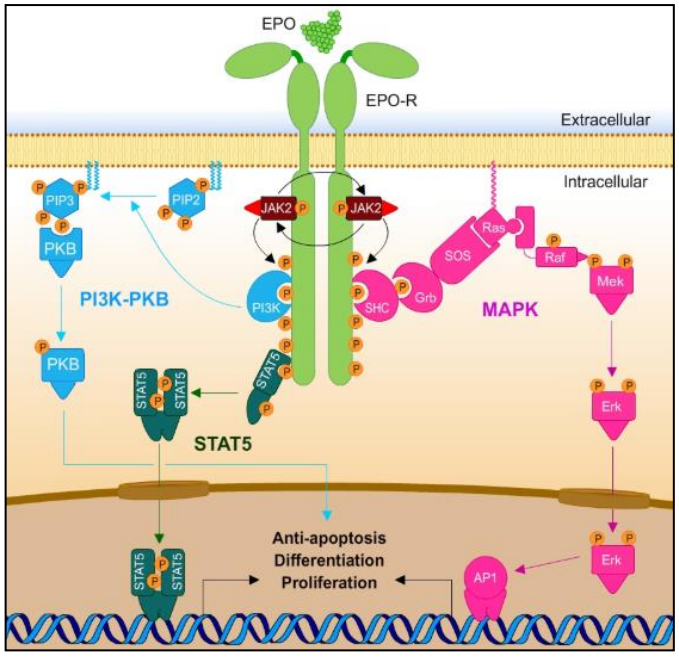

Figure 9. Scheme of Epo receptor signalling. From [116].

EpoR signalling can be inhibited by the cytokine-inducible SH2 protein 3 (CIS3), named also suppressor of cytokine signalling (SOC-3), which is able to bind to phosphorylated EpoR and JAK2 [117]. Finally, the effect of Epo is completed by dephosphorylation of EpoR, executed by the tyrosine phosphatase SHP-1 (Src homology phosphatase-1), followed by internalization of Epo-EpoR complex, which is considered a major mechanism to degrade circulating Epo by erythroid cells [111]. Moreover, the proteasome is involved in controlling

the duration of EpoR signalling by degrading the Epo receptor molecules [118]. Interestingly, while mutations of the extracellular domain of EpoR activate constitutively the receptor due to the formation of an intermolecular disulphide bond, the deletion of an inhibitory signalling element within the carboxy-terminal end of EpoR increases Epo affinity and is associated with erythrocytosis [119].

1.5 Sites of Epo production and Epo-producing cells

In mammals, the main Epo-producing organs are the fetal liver and the adult kidney. In fact, organ ablation studies demonstrated that, while the liver is the main site of Epo production during fetal life, the kidney becomes the predominant source of Epo from birth on until adulthood [120].

Liver

In the fetus, the liver is a suitable organ for Epo production as its structure matches the functional aspects of the fetal circulation. Oxygenated blood coming from the maternal side of the placenta to the fetus needs to pass through the ductus venous, bypassing the liver and flowing directly to the heart. This results in a condition of relative hypoxia of the hepatic tissue, which is thought to be responsible in stimulating Epo production in the fetal liver, that, however, remains significantly lower compared to adult mammals [25]. Moreover, the mammalian fetal arterial blood pO_2 is much lower than that of maternal blood, leading to local hypoxia sensed by hepatic oxygen receptors which increase Epo levels in the serum and amniotic fluid, as measured in sheep and rat fetus [121] [122]. Although evidence are missing, the local hepatic hypoxia together with the placenta aging process have been proposed to trigger the activation of the renal oxygen sensors and the switch of Epo production in the kidney [25]. The induction of hepatic Epo is ensured by the presence of local oxygen tension gradients as demonstrated by *in situ* hybridization studies where Epo mRNA has been found preferentially in pericentral areas of the hepatic lobules, which have lower pO_2 than periportal areas [123]. The discovery of the fetal liver as primary site of Epo production was crucial for the progress of the Epo research field as allowed the cloning of human Epo by preparing Epo cDNA from human fetal liver [10]. Intriguingly, experiments in anephric animals revealed that the adult liver can also contribute to Epo production to an extent of about 5-15%, depending on the severity of the hypoxic stimulus [25]. Generally, the hepatocytes are less sensitive to hypoxia-induced Epo production than the REP cells and this might explain why the adult liver does not compensate for the production of Epo when the kidneys fail [21].

In anaemic rat livers, *in situ* hybridization studies showed Epo mRNA signal predominantly in hepatocytes and, rarely, in interstitial nonparenchymal liver cells [124]. Furthermore, in anaemic transgenic mice carrying the human Epo gene, human Epo mRNA was also detected mainly in hepatocytes and to a lower extent in interstitial liver cells [123]. Additional studies using freshly isolated cells from hypoxic rat livers confirmed the localization of Epo mRNA in hepatocytes as well as showed that Epo mRNA expression from normoxic isolated hepatocytes is stimulated upon hypoxia *in vitro* [125]. Overall, these experimental approaches demonstrated that two different cellular populations, the hepatocytes and non-parenchymal cells express Epo and stimulate Epo synthesis upon hypoxia. The non-parenchymal cell population has been further analysed by Maxwell and collegues using anaemic transgenic Epo-reporter mouse carrying the marker SV40 large T antigen inserted into the 5' untranslated region of the *Epo* gene by homologous recombination into the native Epo locus [126]. By immunolabeling and immunoelectron microscopy, the author could characterize the non-parenchymal cells as Ito cells, hepatic stellate cells also known as lipocytes. These cells have many common features with renal interstitial Epo-producing cells (see below) besides expressing Epo. First, they both are located between the capillaries and the parenchymal cells showing a dense network of processes. Then, they both proliferate and differentiate to myofibroblasts in response to injury and express the surface enzyme ecto-5'-nucleotidase, a fibroblast marker whose synthesis is enhanced in hypoxia [127] [128]. Although cultured Ito cells might provide a suitable model to study the hepatic regulation of erythropoietin in hypoxia, no cell culture lines have been derived so far, neither from hepatic Ito cells nor renal fibroblasts. [126]. In contrast, in 1987 Golberg and colleagues were successful in developing liver-derived cellular models capable to maintain Epo production under culture conditions. These cells, called Hep3B and HepG2, have been generated from human hepatic carcinoma and are able to express Epo mRNA in an oxygen-dependent manner. Since their discovery they became the most widely used *in vitro* models to study Epo regulation [129]. Finally, due to the lack of the feedback regulation of renal erythropoietin during chronic hypoxia (unpublished results from our lab) and their hepatic nature, HepG2 and Hep3B cannot represent an appropriate model to study Epo regulation in the kidney.

In mice, Epo mRNA expression has been detected in the kidneys already from mid-gestation on, whereas in rats renal Epo transcripts increased after birth and hepatic Epo transcripts dramatically decreased in adulthood [130]. In humans, the liver contributes to Epo production not only in fetal, but also in neonatal life and the switch from fetal liver to adult kidney occurs several months after birth [131]. Since the switch from fetal liver to adult kidney occurs in a species-specific manner, it is unlikely that changes in oxygenation happening during birth can drive this phenomenon [128]. How can it be explained that Epo production switches from

fetal liver to adult kidneys? An important aspect to take into consideration is that kidney maturation in many species is not yet complete at birth. Indeed, only when the formation of the organ is reached, the kidney acquires either its functions of excretion and reabsorption or hormone (Epo) production. The latter depends on the kidney oxygen-sensing capacity, which is gained as soon as the structure of the organ is fully mature and completely functional [21].

Kidney

As mentioned before, the kidney is the major Epo-producing organ in adulthood. The reason behind can be found in the observation that the kidney is the main oxygen-sensing organ in the body, in addition to aortic and carotid bodies. Both the aortic and carotid bodies, which are located on the aortic arch and on the carotid artery, respectively, are characterized by the presence of peripheral chemoreceptors, which are able to detect changes in partial pressure of oxygen and changes in partial pressure of carbon dioxide and pH in arterial blood. A decrease in arterial pO_2 is immediately sensed by the chemoreceptors that transmit this information to the respiration control centre of the brain stem which triggers an increase in alveolar ventilation to counteract the low oxygen partial pressure. Interestingly, while the aortic and carotid chemoreceptors are located in a highly perfused tissue, which allows them to sense only changes in partial pressure of oxygen, the kidney is able to detect changes in actual oxygen content of the blood due to its specific organ structure [132] [133]. Moreover, ablation of the arterial chemoreceptors has no significant effect on Epo production in response to hypoxia, suggesting that the regulation of Epo is independent of chemosensors while affected by local oxygen sensing in kidney [134]. The primary functions of the kidney are blood filtering, water and nutrient reabsorption, electrolyte excretion as well as urine production and excretion. The kidney is able to execute these complex functions due to its highly organized anatomical structure and vasculature. The kidney can be divided in three different regions, which are, from outside to inside, the cortex, the medulla and the renal sinus. Cortex and medulla form together the renal parenchyma that includes millions of nephrons, whereas the renal sinus is the cavity below the renal parenchyma that is connected to the outside environment through the hilum. The nephron is the functional unit of the kidney which works as an independent unit producing a small amount of urine. The nephron is composed by a filtering component, namely renal corpuscle, and a tube, known as renal tubule, specialized for reabsorption and secretion, which drains from the renal corpuscle into the collecting duct. Of note, the peculiar architecture of the renal vasculature might explain why the kidney is the ideal organ for local oxygen sensing as well as for the regulation of Epo production. Blood is supplied to each kidney by one renal artery coming from the abdominal aorta and entering the kidney through the hilum, from which the renal

vein leaves the kidney to connect to the inferior vena cava. The renal artery branches in anterior and posterior vessels that run in front or behind the renal pelvis. Within the renal sinus, the arteries divide into interlobar arteries, which enter the renal parenchyma near renal papillae and ascend the medullary pyramids, forming the arcuate arteries that run parallel to the renal capsule giving rise to arches over the medullary pyramids. Arcuate arteries branches into interlobular arteries within the medullary rays, which divide into afferent arterioles that enter the glomerulus and constitute the glomerular capillary network. Blood leaves the glomerulus through efferent arterioles, which separate into peritubular capillaries or descending vasa recta (straight vessels), depending on whether they associate with superficial/midcortical nephrons or juxtamedullary nephrons within the medulla. While the peritubular capillaries supply blood to the cortex, the vasa recta travel parallel to the renal tubule within the medulla and, like the tubule, turn back at different depths towards the corticomedullary junction in the medulla (Figure 10). Venous blood is returned through interlobular veins in the cortex, through arcuate veins in the mid-cortex region and through ascending vasa recta that fuse to arcuate veins [132]. Interestingly, several studies showed that a large oxygen gradient resides along the renal cortico-medullary axis. Such oxygen gradient results from blood flow inhomogeneity between cortex and medulla as well as from the unique renal microvasculature architecture [135] [136].

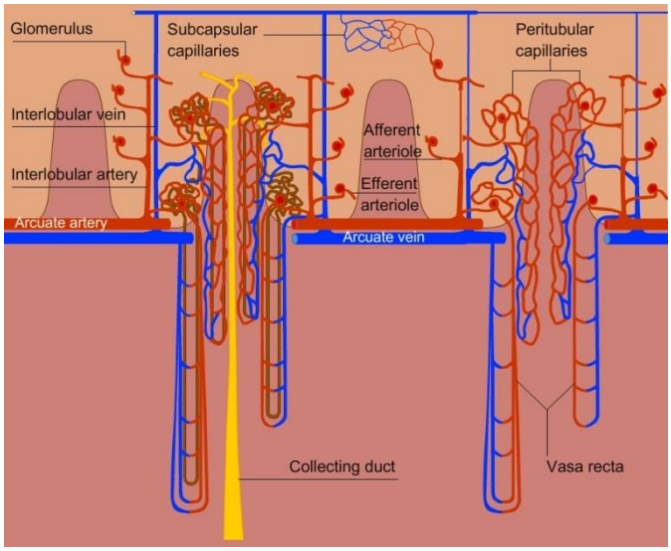

Figure 10. Renal peritubular vascular anatomy. From [137].

While the renal cortex is supplied by 90% of renal blood flow, only 20% of renal blood flow reaches the medulla [138] due to the unique and heterogenous renal vasculature. In addition, descending vasa recta (arterial straight vessels) and ascending vasa recta (venous straight vessels) run in a countercurrent manner, allowing shunt diffusion of oxygen between the arterial and venous vessels, which reduces the tissue pO_2. As a consequence, tissue pO_2 values are comprised between 40 to 70 mmHg in the cortex, 30 to 50 mmHg in the outer

medulla and down to about 10 mmHg near the papillary tip [139]. Intriguingly, the very steep gradient of oxygen tension (40 to 70 mmHg; up to 42 mm Hg per 10 µm) occurring in the renal cortex might explain the oxygen sensitivity of the renal Epo producing cells as well as their particular distribution. Importantly, such renal oxygen gradient is unaffected by changes in organ perfusion as the more blood flow, the more sodium reabsorption through the tubular system, the more oxygen consumption in renal proximal tubule cells but also the more oxygen supply reaches the kidney. Therefore, the ratio between renal blood flow (oxygen supply) and oxygen consumption is kept constant, rendering the kidney the ideal organ to sense small changes in local tissue pO_2 resulting from changes in blood oxygen content rather than blood flow. In fact, the oxygenation of the EPO-producing cells depends mainly on the oxygen carrying capacity of the blood. Moreover, the presence of proximal tubular cells having high oxygen consumption in close proximity to the Epo-producing cells ensures that the pericellular oxygen tension is determined by haemoglobin unloading and not by variations in arterial blood pO_2 due to possible lung function alterations. In this regard, it has been reported that inhibition of sodium reabsorption of proximal tubules decreases hypoxia-induced Epo production, highlighting the strategical location of the Epo-producing cells in the vicinity of the proximal tubule cells [140]. Similarly to the oxygen-sensitive cells of the carotid body, which detect changes in oxygen tension in arterial blood and modulate accordingly the respiration, the renal Epo-producing cells sense changes in local tissue oxygen tensions and stimulate Epo production as shown by studies in isolated perfused kidneys, where Epo mRNA expression in the perfusate was increased in response to changes in oxygen tension [141] [142].

Renal Epo-producing (REP) cells

Attempts to identify and localize the REP cells have been particularly challenging due to the fact that, in contrast to other hormones, Epo is not stored in the cells producing it. While immunoistochemical approaches generated ambiguous results, *in situ* hybridization revealed to be a more reliable method to study the renal Epo-producing cells [21]. Epo mRNA has been localized to peritubular cells in the renal cortex by *in situ* hybridization. The renal Epo-producing cells are peritubular, interstitial, fibroblast-like cells located along the corticomedullary oxygen gradient at the border between cortex and medulla (juxtamedullary cortex), which appears to be the perfect location to sense small variations in local oxygen tension due to presence of local oxygen gradients [143] [144] [145]. In fact, oxygenation of REP cells depends on the O_2 diffusion driven by pO_2. During inspiratory hypoxia or anaemia, pO_2 falls, thus, less oxygen diffuses to the juxtamedullary cortex leading to the increase of REP cells number (Figure 11) [133].

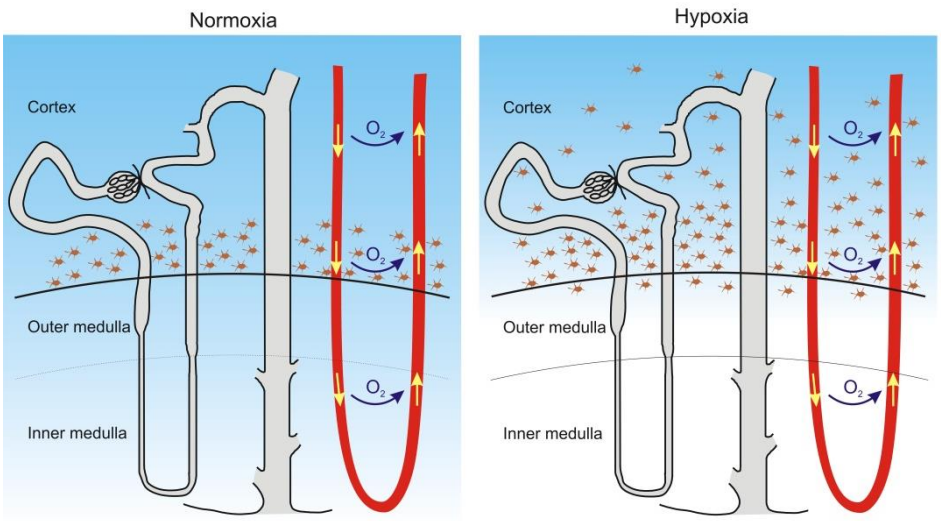

Figure 11. Schematic representation of the renal tubular system and the countercurrent oxygen exchange, forming the cortico-medullary oxygen gradient (blue to white background). In hypoxia, the oxygen gradient is shifted to the outer cortex and the number of REP cells at the border between medulla and cortex increases. From [133].

The first study demonstrating that REP cells are peritubular interstitial fibroblasts, localized mainly in the cortex and the outer medulla was performed by Maxwell and collegues in 1993. They generated a genetically modified mouse, in which the SV40 large T antigen was under the regulatory control of the *Epo* gene locus, allowing the identification of REP cells by immunohistochemistry using anti-T antigen antibodies. In response to hypoxia or anaemia, the expression of Epo/SV40 large T antigen was induced and the accumulation of SV40 large T antigen was detected in nuclei of cortical peritubular interstitial fibroblasts, expressing the fibroblast marker 5'-ectonucleotidase (CD73), but not in other types of peritubular cells [146]. This elegant work proved that renal fibroblasts are the most promising candidates of renal Epo-producing cells among the other proposed cell types including tubular epithelial cells, glomerular mesangial cells and interstitial fibroblasts [147]. Successively, the Yamamoto group was able to unequivocally localize the REP cells and confirm the previous *in vivo* findings of Maxwell et al., by two complementary approaches, the BAC transgenic mice and the green fluorescent protein (GFP) reporter knock-in mice. The BAC transgenic mice (Tg-EpoGFP) carry a transgenic construct in which GFP expression is directly controlled by the 180 kb regulatory region of the mouse *Epo* locus by replacing the region spanning exon II to intron IV of the *Epo* gene, were sufficient to recapitulate kidney and liver specific Epo expression and allowed the labelling of Epo-producing cells by GFP expression. On the other hand, the GFP knock-in mice (KI-EpoGFP) ensured more specific targeting of the REP cells as GFP cDNA was knocked-in the endogenous *Epo* gene leading to GFP expression controlled by endogenous Epo regulation. Both strategies were able to detect only few GFP-positive interstitial cells in normoxia, while in anaemia or hypoxia the number

of GFP-positive interstitial cells significantly increased, showing appropriate oxygen-dependent regulation. Moreover, these GFP-positive cells, located in the renal interstitium and induced upon hypoxic or anaemic stress, are fibroblast-like cells expressing the fibroblast markers CD73 and platelet-derived growth factor receptor β (PDGFR-β). These cells have dendrite-like processes and express also neural genes like microtubule-associated protein 2 (MAP2), nerve growth factor receptor (NGFR) and neurofilament light peptide (NFL). In contrast, REP cells showed no staining for blood vessel (platelet endothelial cell adhesion molecule 1, PECAM or cluster of differentiation 31, CD31) or macrophages (macrophage antigen 1, Mac1) (Figure 12) [148] [149].

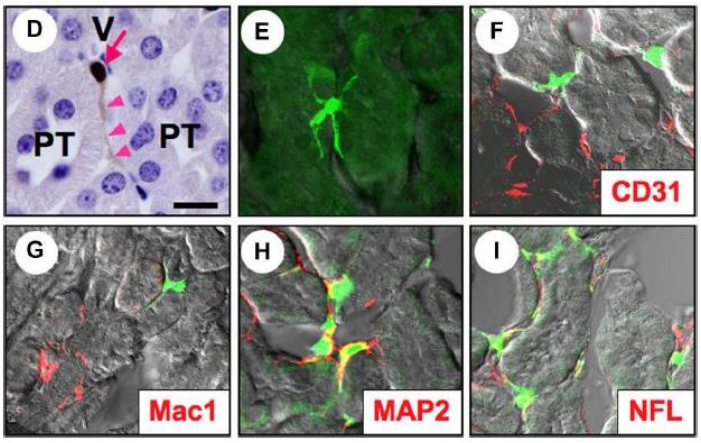

Figure 12. Localization and characterization of Epo-producing cells in renal interstitium from Epo-GFP transgenic mice. GFP expression (brown) was detected in the peritubular interstitial cells (arrows in D) with long projections (arrowheads in D) between the proximal tubules (PT) and vessels (V). REP cells (green) from anemic kidneys of transgenic mice costained with cell lineage markers (red) were negative for CD31 (F) and Mac1 (G), but positive for MAP2 (H) and NFL (I). From [149].

As mentioned before (Figure 3), Suzuki et al. observed Epo production in neural and neural crest cells of E8.5–E11.5 embryos, supporting the hypothesis of neuronal origin of REP cells as already suspected by a previous work of Asada and collegues, who discovered that most of interstitial fibroblasts in the kidney derived from myelin protein 0 (P0)-positive neural crest cells and that less than 20% of P0 lineage-derived cortical fibroblast co-express Epo, CD73 and PDGFR-β in Tg-EpoGFP mice [39] [150]. Interestingly, the number of GFP-positive cells in Tg-EpoGFP or KI-EpoGFP are considerably lower than those of Inherited Super-Anemic Mice (ISAM), which are transgene-rescued mice carrying a 8-kb transgene containing the liver but not kidney Epo regulatory elements, sufficient to rescue the embryonic lethality of Epo null mice ($Epo^{GFP/GFP}$). ISAM mice show Epo production restricted to the liver and develop adult onset severe chronic anaemia caused by defects in renal Epo synthesis, whereas Epo-producing cells in ISAM kidneys are well labelled by GFP expression under the regulation of Epo gene locus [151] [147]. While the above mentioned Tg-EpoGFP and KI-EpoGFP mouse models have been particularly useful to investigate the cells that acutely

express Epo, demonstrating that the EpoGFP-expressing cells consist in a small fraction of the total REP cells, they were not able to track the fate of the Epo-producing cells over time as the GFP expression is limited to the timeframe of active Epo production. Therefore, to identify the total population of REP cells, the Yamamoto group crossed the tdTomato reporter mouse line (R26RtdTomato) to new transgenic mice, in which the Cre-recombinase was expressed under the control of the 180 kb regulatory region of the mouse Epo locus (Tg-EpoCre). This mouse model (R26RtdTomato:TgEpoCre) allowed for the detection of Epo-Cre positive cells through the expression of red fluorescence in cells that expressed Cre and thus Epo at least once in their life [152] [153]. When R26RtdTomato:TgEpoCre mice were crossed to ISAM animals leading to the generation of ISAM-REC, the EpoCre transgene was induced by chronic severe anaemia typical of the ISAM mice and tdTomato fluorescence was detected in almost all fibroblast-like interstitial cells expressing both PDGFRβ and CD73 in cortex and outer medulla, representing all the cells capable to produce Epo (total REPCs), which appear to be, indeed, more abundant than those in Tg-EpoGFP mice (Figure 13) [153].

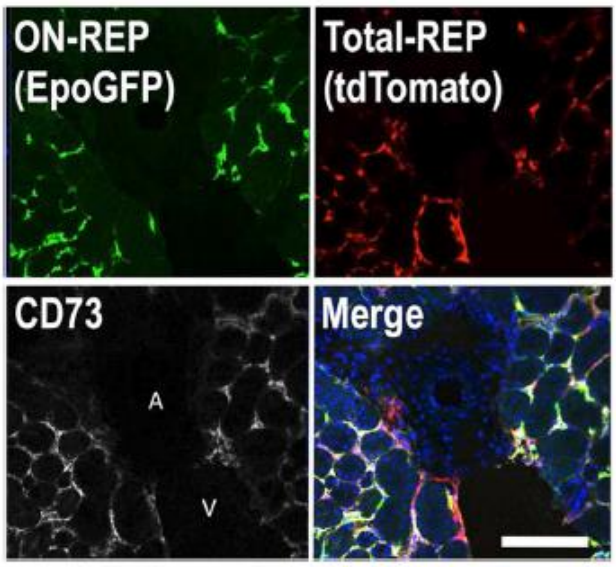

Figure 13. Renal Epo-producing (REP) cells are peri-capillary fibroblast-like CD73-positive cells. ON-REPs expressing EpoGFP (green) and CD73 (white) within the total REP population expresses tdTomato (red) from a kidney section of ISAM-REC. The fibroblast-like REP cells are distributed in peri-capillary but not in peri-arterial interstitial spaces of the kidneys. Nuclei are stained with DAPI (Merge). Abbreviations: A, artery; V, vein. Scale bar 100 μm. From [147].

Intriguingly, Yamazaki et al. observed that REP cells distribute around the peritubular capillaries but not around the larger vessels and calculated that approximately 10% of tdTomato positive cells express active Epo, thus being in a resting phase from Epo production despite the severe anaemic conditions in ISAM mice.

Remarkably, a recent work from the Haase group found that REP cells of the adult mouse kidney were derived from FoxD1-expressing stromal cells, which surround the cap mesenchyme during kidney development, giving rise to cortical and medullary renal interstitial fibroblast, pericytes, mesangial cells and vascular smooth muscle cells. Furthermore, the authors suggested that the renal Epo-producing cells, derived from FOXD1-expressing stromal progenitors were heterogeneous in response to stimuli of Epo production [154].

Several efforts have been made to isolate REP cells from mouse kidneys in order to obtain an *in vitro* kidney-derived cell culture model for oxygen-regulated Epo expression. None of these attempts was able to provide a reliable kidney-derived cell line with hypoxic Epo regulation as renal interstitial fibroblasts tend to lose the capability to produce Epo *in vivo* and when induced to proliferate *in vitro* as the *Epo* locus is only transiently activated by oxygen-dependent stimuli.

Brain

Epo and EpoR are expressed in the CNS already during human fetal development between 7 and 16 weeks after gestation, with increasing levels from 8 to 24 weeks [155]. Epo and EpoR mRNA have been detected in the brain of mice [60], monkeys and humans [156]. In human brain, the expression of Epo has been localized to the temporal cortex, hippocampus and amygdala [157] as well as to the CSF of human adults [158] and neonates [159]. The main cellular sources of brain-derived Epo are astrocytes [156] and neurons [160], whereas it remains unclear whether brain endothelial cells are capable to produce Epo [157]. EpoR mRNA has been found in a broad variety of cerebral cells, including neurons and astrocytes [160], endothelial cells [59] [161] as well as microglial cells [162]. The endogenous Epo production in brain, astrocytes and neurons occurs locally, suggesting that Epo may act outside the bone marrow in a paracrine or autocrine manner in response to hypoxia [156]. Importantly, Epo has been shown to cross the BBB despite its low permeability thus representing a new frontier in the treatment of brain disorders [163] [164].

While the kidney produces Epo only temporarily during hypoxia, neuronal Epo expression is upregulated upon a hypoxic stimulus and the increase in Epo levels in the brain is continuous [165]. Hypoxic Epo transcripts increase between 3- and 20-fold in the brain compared to the 200-fold induction in the kidney. Therefore, the hypoxic stimulation of the *EPO* gene in the brain seems to be similar to the kidney even though the Epo mRNA levels in the brain are lower [157]. Intriguingly, primary mouse astrocytes showed 100-fold Epo mRNA levels in response to hypoxia and also neurones were reported to stimulate the transcription of the

Epo gene in hypoxia [166]. Additionally, hippocampal neurons *in vitro* showed hypoxia-mediated upregulated EpoR mRNA levels [167]. On the other hand, proinflammatory cytokines (IL-1β, IL-6, or TNF-α) decreased both Epo and EpoR expression in astrocytes, whereas human neurons cultured in presence of TNF-α showed increased expression of EpoR [162]. Anaemia and ischemic conditions were both reported to enhance EpoR expression *in vivo* by rendering the neuronal cells more sensitive to Epo response [89]. Notably, neuronal Epo and EpoR proteins have a smaller molecular weight than their counterparts in the periphery as they possess different amount of sialic acid [168] [169].

Epo has been suggested to have a physiological role in the brain as Epo and EpoR are highly expressed in the embryonic brain [170]. $Epo^{-/-}$ and $EpoR^{-/-}$ mice, which die *in utero* due to the lack of definitive erythropoiesis and severe anaemia, have been reported to exhibit a thinning of the neuroepithelium and smaller brain size with incomplete closure of the neural tube at E10.5 as well as increased apoptosis of neuronal cells, harvested from EpoR null mice at E10.5 that did not show yet a significant anaemic phenotype, indicating a role of erythropoietin in brain development [171]. The physiological effect of Epo in the brain has been evaluated in neurons both, *in vitro* and *in vivo*. Several studies suggested that brain-derived Epo is a physiological neurotrophic factor before, during or after neuronal injury [172]. The first evidence for Epo-mediated neuroprotection was provided by the *in vivo* work of Sakanaka et al. who demonstrated that infusion of exogenous Epo (2.5–25 units/day) in gerbils with mild (2.5 mins) brain ischemia is neuroprotective against ischemic damage of hippocampal CA1 neurons [173]. Furthermore, hypoxia-induced Epo has been shown to promote the production of neuronal progenitors acting on forebrain neural stem cells [174]. Thus, brain-derived Epo may represent an endogenous protective factor for neurons against both mild ischemia and tissue hypoxia [157]. On the cellular level, brain-derived Epo has been suggested to be involved in the regulation of calcium flux in neuronal cells [175] as well as in the stimulation of dopamine release [176]. Thus, it is believed that Epo might promote neuronal function and viability through activation of calcium channels and release of neurotransmitters [157]. In addition, the physiological effect of brain-derived Epo has been linked to angiogenesis: brain capillary endothelial cells have been showed to express two forms of EpoR mRNA [161]. Because endothelial cells express EpoR and react to Epo, it is believed that endothelial cells and haematopoietic cells derived from the same mesenchymal precursor, the so-called haemangioblast [177], which clarifies why endothelial cells possess the EpoR [178]. Furthermore, studies performed in mice with cerebral infarct have shown that brain endothelial cells display a staggered induction of EpoR and Epo, with Epo following EpoR protein [89]. However, it remains unclear whether the production of Epo attributed to brain endothelial cells contributes to the paracrine Epo response in human

neuronal cells [172]. Importantly, the endothelial response to Epo leads not only to endothelial cell proliferation and angiogenesis, but also to increased expression of endothelial nitric oxide synthase (eNOS) that produces the vasodilator nitric oxide (NO) improving the oxygenation of the hypoxic tissue [69]. Of note, it has been shown that Epo promotes maturation and differentiation of oligodendrocytes and the proliferation of astrocytes *in vitro* [179].

In summary, tissue hypoxia and cerebral ischemia stimulate the transcription of oxygen-regulated genes such as Epo and vascular endothelial growth factor (VEGF) in neuronal cells through hypoxia-inducible factors. While Epo acts mainly on neurons triggering neuroprotection and apoptosis prevention, VEGF induces proliferation of endothelial cells and new vessels formation (angiogenesis), which decreases the oxygen diffusion distance and thus increases the oxygenation of the hypoxic tissue [180].

Until recently, it was thought that the anti-apoptotic function of brain-derived Epo involved the same erythropoietic Epo/EpoR axis [181]. However, in 1990, Anagnostou and colleagues performed receptor-protein crosslinking studies and observed that the EpoR in endothelial cells was constituted by only one associated protein of ~45 kDa [182], rather than two larger ones of ~110 kDa and ~95 kDa, typical of the erythroid EpoR [183]. In addition, such endothelial EpoR was present at much higher density but at lower affinity for Epo, compared to its counterpart in erythroid cells (~5 nM versus ~200 pM, respectively) [182]. Successively, neuronal-like PC12 cells were reported to possess EpoR with very low Epo affinity (~20 nM), which also gave a single crosslinked product but different from the endothelial cells [161]. The existence of EpoR with nanomolar affinities for Epo confirmed that Epo may act locally in the brain via an autocrine or a paracrine way, as the circulating Epo levels are too low (1-5 pM) to signal through the endothelial EpoR (~2 nM) [181]. Taken together, these findings suggested that a different type of EpoR exist and that it should contain the erythroid subunit as increased tissue EpoR mRNA was found following ischemia whereas anti-EpoR antibodies abolished neuroprotection [184]. Studies in the IL-3-dependent murine cell line, Baf3, showed association of EpoR with the β common receptor subunit βCR, which is also present in interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GMCSF) and IL-5 receptors (Figure 14) [185]. Although it remains unclear whether the βCR subunit is associated with Epo-R [186], βCR knock-out mice confirmed that the βCR subunit is required for tissue protection as shown by the fact that Epo had no protective effect in the presence of the EpoR. However, a role for homodimeric EpoR in tissue protection cannot be excluded yet. Intriguingly, Brines et al. postulated that this new non-erythroid receptor, able to specifically trigger tissue protection, consist of one EpoR monomer and a dimer of the β common receptor (βCR) [91]. Brines and colleagues have

proposed that βCR exists as a preformed homodimer consisting of intertwined extracellular domains and that Epo induces the formation of a heterotrimer composed by EpoR monomer in the centre and βCR homodimer at the sides of EpoR. (Figure 15) [181]. The formation of such Epo-EpoR-βCR complex results in the interaction of the intracellular domains and subsequent activation of the Janus tyrosine kinase 2 (JAK2), which then initiates signalling cascades mediating tissue protection.

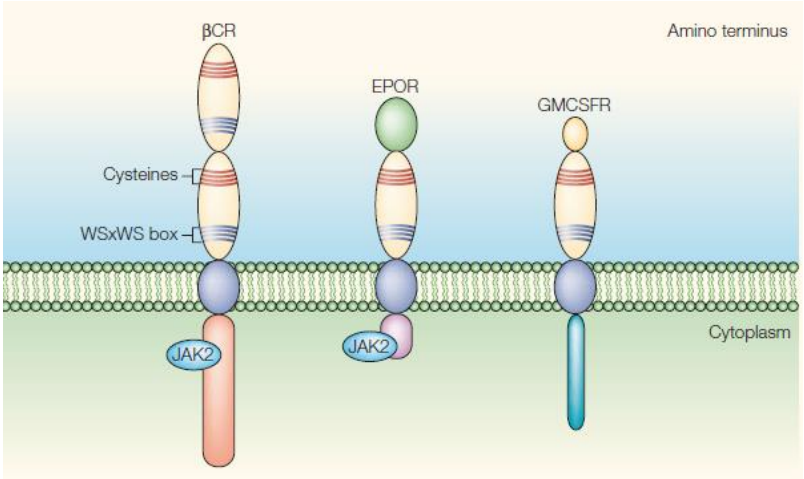

Figure 14. Structure of Epo receptor subunits mediating neuroprotection, erythropoiesis and cytoprotection. Epo-R belongs to a subfamily of the type I cytokine receptor superfamily, which also includes the β common receptor (βCR) and the granulocyte-macrophage colony-stimulating factor (GMCSF) receptor. These receptors share several structural features: the "tryptophan-serine-any amino acid-Trp-Ser" (WSxWS) motif at the extracellular domain, near the plasma membrane as well as 4 cysteine residues more distally distributed. The intracellular domain of the βCR is a signalling component shared also by GMCSF and IL-3 and IL-5. From [181].

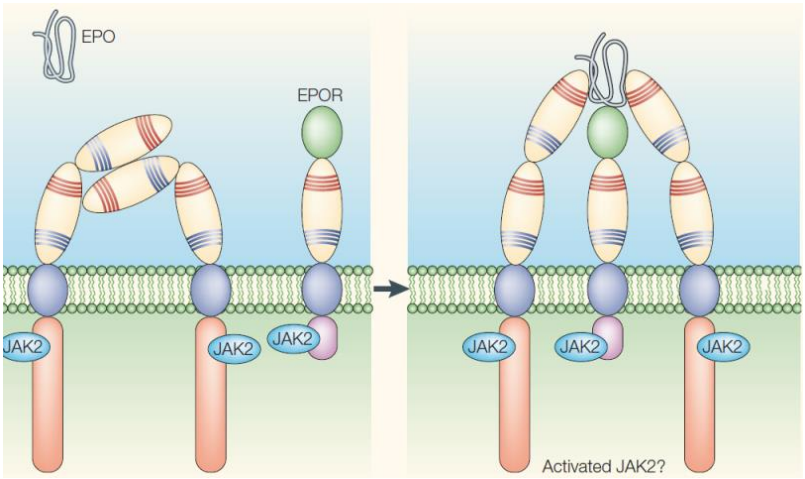

Figure 15. Proposed structure of the Epo-mediated heterotrimer involved in tissue protective signalling. From [181].

As shown in Figure 16, the tissue-protective molecular pathway triggered by Epo is partially similar to the erythropoietic pathway as it involves the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and the protein kinase B (PKB/Akt)-nuclear

factor-κB (NF-κB) pathways. In contrast to the erythropoietic pathway, the recruitment of the transcription factor STAT5 is still a matter of debate as *in vivo* evidence is missing.

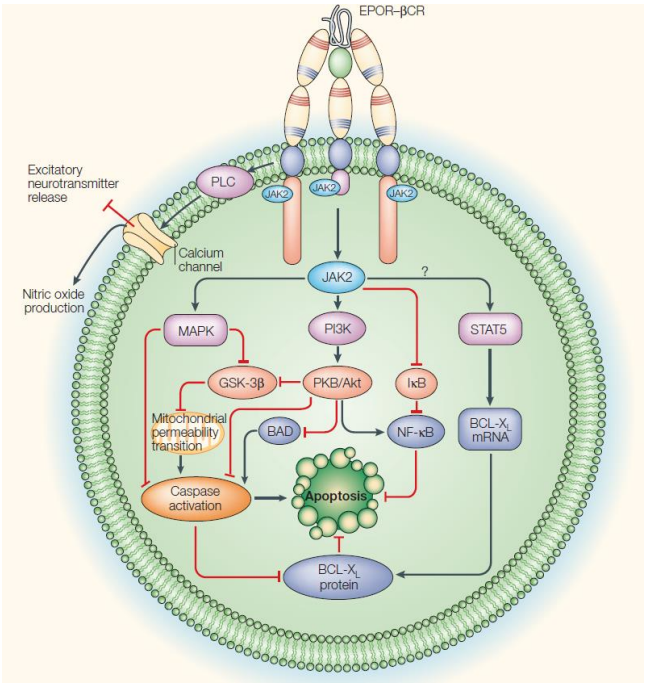

Figure 16. Epo mediates a signalling pathway involved in tissue protection. The black lines indicate the pathways shared by the erythropoietic and the tissue protective signalling whereas the red lines highlight the specific secondary messengers leading to tissue protection. Note that Epo modulates also the activity of calcium channels through phospholipase C (PLC), reducing the release of excitatory neurotransmitters and enhancing the nitric oxide production. From [181].

The key secondary messengers of the tissue protective pathway include the glycogen synthase kinase 3β (GSK3β) that in turn inhibits the mitochondrial permeability transition pore [187] that is implicated in cell death through cell caspase activation; the inhibitor of κB (IκB) that is directly inhibited by JAK2, leading to the activation of NF-κB and the consequent transcription of anti-apoptotic genes; the protein kinase B (PKB/Akt) that has a direct inhibitory effect on GSK3β, and B-cell leukaemia/lymphoma (BCL) family 2-associated death protein (BAD), a pro-apoptotic protein. Finally, a specific tissue-protective function of MAPK and PKB/Akt is the indirect prevention of the caspase activation through the increase of anti-apoptotic proteins belonging to the BCL family [181].

The discovery of a putative Epo heteroreceptor with lower affinity for Epo than the bone marrow and with tissue-protective functions opens new horizons toward clinical applications. In fact, the possibility to engineer non-erythropoietic Epo molecules having only a tissue-protective activity is tempting. So far, three approaches were successful [181]. First, the removal of sialic acid on Epo resulted in asialo-erythropoietin with a half-life of only several minutes *in vivo,* able to exclusively interrupt apoptosis without erythropoietic effects [188].

Second, mutations at either the EpoR binding site or the intracellular domain activating the signaling cascade in Epo produce molecules that are unable to bind to EpoR homodimer and that trigger tissue-protective effects [189]. Third, carbamylation of erythropoietin confers full non-erythropoietic tissue protection [189].

In conclusion, given the high potential of neuroprotective Epo for the treatment of several neuropathologies caused by tissue injury, clinical trials need to be carried-out in order to verify the appropriate dose-response and all the possible side effects of Epo therapy.

1.6 Hypoxia

The evolution of atmospheric oxygen (O_2)

Oxygen is essential for survival of animals, plants, aerobic organisms and has a central role in driving the evolution of life on Earth [190]. The composition of the terrestrial atmosphere has markedly changed during ~4,500 million years since our planet formed, strongly influencing the development of living organisms [191]. For the first half of Earth's history, no oxygen was present on our planet, which was inhabited only by single cell-organisms. Indeed, our life-giving gas started to appear only ~2.4 billion years ago, when the so-called Great Oxygenation Event (GOE) took place. There is evidence that the first photosynthetically active single cell-organisms evolved about 3.0 billion years ago. What triggered the Earth oxygenation? Ancestral microorganisms, the cyanobacteria, developed the ability to utilize sunlight energy and water to fuel their own metabolism producing sugars through the process of photosynthesis resulting in the generation of molecular oxygen (O_2). Nowadays, it is believed that cyanobacteria augmented the oxygen levels in the atmosphere during the GOE, transforming the Earth's atmosphere and contributing to the beginning of life [192]. Since O_2 is highly reactive and a toxic by-product of cyanobacteria's photosynthesis, initially, it was eliminated from the atmosphere via oxidation of inorganic minerals (e.g. iron). Then, minerals became saturated with oxygen and the oxygen levels rose up, especially due to the active metabolism of cyanobacteria [193]. In presence of oxygen, most of unicellular anaerobic organisms could not survive but complex organisms, the eukaryotes, formed through the symbiosis with different bacteria families. By engulfing an aerobic prokaryote and establishing an endosymbiotic relationship [194], eukaryotes evolved special membrane-enclosed compartments, the mitochondria, able to either resist to the toxic effect of oxygen or exploit its chemical energy to generate the high energy molecule adenosine triphosphate (ATP) in a much more efficient manner than the anaerobic metabolism. In fact, while anaerobic glycolysis leads to the production of only 2 ATP molecules per glucose molecule, the aerobic respiration generate virtually 30 ATP molecules per each metabolized glucose via glycolysis and citric acid cycle [195]. The development of mitochondria as energy cell factories fuelled by oxygen ensured the survival of eukaryotes, which were evolutionary selected to live in oxygen-enriched atmosphere. The abundant oxygen supply assured by photosynthesis and the oxidative phosphorylation through mitochondria matched and favoured the evolution of complex multicellular organisms (*Metazoa*), plants and animals [196].

(Patho-)Physiological Hypoxia

The cells of our body are exposed to different oxygen levels depending on a wide range of factors: organ anatomy, blood perfusion, oxygen consumption of the specific tissue as well as the physiological condition (e.g. exercise, rest, and high altitude). As shown in Table 1, cells belonging to distinct organ/tissue are exposed to diverse physiological oxygen tensions, which are lower than 160 mmHg (the partial pressure of O_2 in the air at sea level, equivalent to 21% O_2) [197]. Oxygen is distributed in tissues through gradients of partial pressure of O_2 (pO_2). In blood capillaries, the pO_2 gradients occur longitudinally along or radially from the vessels, varying between approximately 100 mmHg in arterial blood and 40 mmHg in mixed venous blood. In the kidney, the partial pressure of O_2 drops from 50-70 mmHg in the cortex to 10-20 mmHg in the medulla [198]. As many cell types are not able to store large amounts of oxygen, they are particularly sensitive to any condition in which the oxygen demand exceeds the oxygen supply (hypoxia) [199]. The insufficient availability of molecular oxygen within the cellular microenvironment causes hypoxia, which represents a harmful threat for the physiological functions of our body [190].

Organ/Tissue	**pO_2 (mmHg)**
Air (sea level)	160
Inspired air (tracheus)	150
Air in the alveoli	110
Arterial blood	100
Venous blood	40
Brain	33.8 ± 2.6
Lung	42.8
Liver	40.6 ± 5.4
Kidney cortex	72 ± 20
Kidney medulla	10 - 20
Muscle	29.2 ± 1.8
Cell	9.9 - 19

Table 1. Mean values of oxygen partial pressure (pO_2) in the microenvironment of different human tissues. Adapted from [197].

In healthy humans, physiological hypoxia is found in several processes including new blood vessel formation in adulthood (angiogenesis) as well as during development

(vasculogenesis), wound healing and the adaptation to exercise and high altitude. However, tissue hypoxia is also related to diverse pathophysiological conditions such as atherosclerosis and stroke, where the vascular occlusion impedes oxygenation causing tissue ischemia; chronic inflammation, in which the increased metabolism and oxygen demand of the inflamed tissues cause hypoxia; and cancer, where the tumour growth augments the oxygen consumption, leading to hypoxia [190].

As multicellular organisms need a constant supply of oxygen for their survival, they evolved specific mechanisms based on systemic and cellular responses to adapt to hypoxia. The mammalian systemic response takes place when the entire organism is exposed to low pO_2 as for example at high altitude and consists of a series of changes involving mainly the respiratory and cardiovascular systems. The cellular response concerns all cells of our body, which are able to respond to hypoxia, and includes the activation of oxygen-sensitive pathways within the cell, leading to the stabilization of the transcriptional regulator known as the hypoxia-inducible factor (HIF). The systemic and cellular responses are certainly linked mechanisms that developed during evolution not only to adapt to hypoxia but especially to maintain O_2 homeostasis [200].

1.6.1 Systemic response to hypoxia: adaptation to high altitude

High altitude is defined as elevations above 1500 m sea level at which the atmospheric pressure (P_{ATM}) declines exponentially [201]. The absolute concentration of each gas composing the atmospheric air remains unchanged with increasing altitude [198]. At 5500 m (base camp used in ascents to Mount Everest) P_{ATM} is 380 mmHg that is half the value at sea level (760 mmHg) and pO_2 is 80 mmHg (21% of 380 mmHg). However, our body can still meet the oxygen demands of healthy people at such elevation. At the peak of Mount Everest, the highest point on Earth (8848 m above sea level), P_{ATM} is only 255 mmHg and pO_2 is only 53 mmHg and there hypoxia becomes a severe problem. While ascending a mountain, our body reacts to reduced ambient pO_2 by activating compensatory responses, which varies between individuals. An intrinsic protective mechanism is found in the sigmoid-shape curve of haemoglobin (Hg), which ensures that oxygen saturation of haemoglobin in arterial blood decreases little at altitude up to 3000 m as pO_2 is between 80 and 60 mmHg corresponding to the relatively flat portion of the O_2–Hb dissociation curve, allowing oxygen content in arterial blood to be slightly affected. However, with elevation, the oxygen uptake by the pulmonary-capillary blood gradually slows down as the alveolar-capillary pO_2 gradient at the beginning of the capillary falls. As a consequence, oxygen deficiency in arterial blood or insufficient loading of haemoglobin (hypoxemia) causes a reduction in the absolute O_2 transport rate. However, our body is capable to compensate for the reduced availability of

oxygen at altitude through short-term and long-term adaptation mechanisms. Their effects are summarized in Table 2 and discussed below.

Short-term adaptation to high altitude

During the first hours at high altitude, our body responds to hypoxemia by increasing the heart rate (tachycardia) so that tissues are faster perfused and by enhancing the respiratory frequency or the tidal volume (hyperventilation) (Table 2). As previously mentioned, peripheral chemoreceptors, located in carotid and aortic bodies, are able to sense a reduction in pO_2 in arterial blood as well as signal to cells in the medulla oblongata (brainstem) to increase ventilation and heart rate. The peripheral chemoreceptors contain chemosensitive cells called type I or glomus cells with neuronal features, able to release neurotransmitters including catecholamines and acetylcholine [132]. Mechanistically, low pO_2 inhibits O_2-sensitive K^+ channels located at the plasmamembrane of the glomus cells, leading to membrane depolarization that stimulates the opening of voltage-gated Ca^{2+} channels increasing the concentration of cytosolic Ca^{2+} [200]. The augmented amount of cytosolic Ca^{2+} provokes neurotransmitter release and consequent activation of either the carotid sinus nerve in carotid bodies or the vagus nerve in aortic bodies, which both signal to the cardiorespiratory centre of the medulla oblongata to increase ventilation. Importantly, hyperventilation increases pO_2 but at the same time causes respiratory alkalosis (↑ pH, ↓ pCO_2) as the exhalation of CO_2 occurs faster than its production. Respiratory alkalosis is an inhibitory stimulus for the peripheral and central chemoreceptors, which decreases ventilation, bringing the body close to the physiological ventilation rate. Over a prolonged exposure at high altitude, CO_2 accumulates, normalizing respiratory hypocapnia (↓ pCO_2) and pH decreases in cerebrospinal fluid (CSF) to counteract respiratory alkalosis. In case the pH in CSF lowers too much, central chemoreceptors sense the increased $[H^+]$ and stimulate ventilation to reduce the CO_2 and the H^+ levels. In addition to the respiratory effects, peripheral chemoreceptors exert also cardiovascular effects: tachycardia, increased cardiac output (↑ CO) and pulmonary vasoconstriction [132]. Pulmonary vasocontriction may occur via a direct action of low pO_2 on the pulmonary vascular smooth-muscle cells with a molecular mechanism similar to the one described above for the glomus cells, where hypoxia inhibits K^+ channels and, ultimately, releases Ca^{2+} from the intracellular storages to trigger myocyte contraction and vasoconstriction of the lung tissue to divert the blood flow away from the underventilated alveoli toward the ventilated alveoli. Of note, our body adapts to this compensatory mechanism in order to correct the ventilation/perfusion mismatch occurring in both underventilated and ventilated alveoli. Pulmonary vasoconstriction increases the blood pressure within the arteries of the lungs and, thus, the resistance of the pulmonary vasculature rises, causing right ventricular hypertrophy [132].

Long-term adaptation to high altitude

While vasoconstriction occurs in the pulmonary circulation, in the systemic circulation, blood vessels vasodilate to optimize the oxygen delivery to the metabolizing cells of the peripheral tissues. Particularly, vasodilation takes place within the endothelial smooth muscle cells in the following way: nitric oxide (NO), produced from L-arginine in endothelial cells of blood vessels by nitric oxide synthase (NOS), activates guanylate cyclase (GC) that converts guanylate triphosphate (GTP) to cyclic guanylate monophosphate (cGMP). Through cGMP-dependent protein kinase G several phosphorylation events occur until the intracellular concentration of Ca^{2+} decreases and the relaxation of smooth muscle cells take place. Moreover, prolonged high altitude causes a decrease of heart rate (HR) and cardiac output to sea levels values, which, initially, leads to reduced oxygen delivery but, then, is compensated by vasodilation of peripheral vessels that maximize the oxygen distribution to the tissues. Another cardiovascular effect caused by high altitude is tissue angiogenesis leading to increased vascularity, triggered by growth factors released by hypoxic tissues such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [132] [202]. In the lung, angiogenesis enhances the capillary surface, improving oxygen diffusion and counteracts the effects of pulmonary vasoconstriction (↓ blood flow and ↑ vascular resistance), leading to an increase in pulmonary diffusing capacity [132].

Short-term adaptation	Long-term adaptation
↑ Ventilation	↑ Epo, ↑ RBC, ↑ [Hb], ↑ Hct,
Respiratory alkalosis (↓ pO_2, ↑ pH)	Systemic vasodilation, ↓ HR, ↓ CO
Tachycardia (↑ CO, ↑ HR)	↑ Angiogenesis, ↑ VEGF
Pulmonary vasoconstriction ↑ vascular resistance	↑ 2,3 BPG, ↓ O_2 affinity of Hb

Table 2. Overview of the physiological changes occurring during short-term and long-term adaptation to high altitude. The short-term adaptation indicates a period from minutes to hours whereas the long-term adaptation refers to a prolonged exposure from few days to weeks. Abbreviations: RBC= red blood cells; [Hb]= haemoglobin concentration; Hct= haematocrit; HIFs= hypoxia inducible factors; HR= heart rate; CO= cardiac output; VEGF= vascular endothelial growth factor; 2,3 BPG= 2,3 bisphosphoglycerate.

Moreover, hypoxia stimulates the RBCs to produce more 2,3 bisphosphoglycerate (BPG) and its accumulation (↑ 2,3 BPG) reduces the affinity of haemoglobin for oxygen as 2,3 BPG binds the allosteric site of haemoglobin, destabilizing the interaction with oxygen and promoting oxygen release.

The kidneys also play a role in the long-term adaptation to altitude: they decrease the rate of acid secretion to the lumen of the renal tubules (↑ $[H^+]$ in blood) and increase the excretion of bicarbonate in the urine (↓ $[HCO_3^-]$ in blood) to counteract the respiratory alkalosis by lowering the alkali blood pH towards more physiological values (Table 2).

Although the mechanism remains unclear, it has been demonstrated that hypoxic stimulation of the carotid bodies and increases sodium and water excretion in the kidneys causing diuresis (↑ urinary flow) and reduced plasma volume until the haematological response takes place [203].

Altitude Sickness

When ascending rapidly to high altitude, hypoxia can cause initially mild symptoms in susceptible individuals including headache, fatigue, nausea and decline in cognition, which are progressive with increasing altitude. Ascents can develop acute mountain sickness (AMS), a clinical condition comprising more severe symptoms such as dizziness, dyspnoea, sleep disturbance, peripheral oedema, vomiting, which develop within the first days. During the acclimatization phase, a series of side effects might appear like fatigue, shortness of breath (dyspnoea) and alternative periods of cessation of breath (apneas) caused by the inhibition of hyperventilation due to too low pCO_2 (hypocapnia).

In the brain, vasodilation of cerebral vessels result in brain swelling and headache and increased capillary pressure, which can provoke capillary leakage and disruption of the blood brain barrier (BBB), leading to accumulation of fluid in the brain causing, ultimately, cerebral oedema. In the lung, an increase in vessel permeability (↑ VEGF) and pulmonary transmural pressure lead eventually to damage of capillaries, accumulation of liquids and, ultimately, high altitude pulmonary oedema. Both pulmonary and cerebral oedema can be fatal if not properly diagnosed and treated. Certainly, the most effective treatment for AMS is descending to lower altitude and providing oxygen by portable hyperbaric chambers to the sick subjects. Protective factors against AMS include, primarily, slow ascent (no more than 300 to 600 m per day) to ensure a gradual acclimatization to progressive altitude, and then, if symptoms occur despite slow ascent, acetazolamide (diuretic and inhibitor of carbonic anhydrase) and dexamethasone (corticosteroid that counteracts the cause of cerebral oedema) can be administered for prophylaxis or treatment of AMS [204] [132]. After prolonged residence at high altitude, chronic mountain sickness (CMS) may develop, mainly caused by the erythropoietic response promoting overproduction of red blood cells (RBC), reaching a very high haematocrit (Hct > 65%). Polycythaemia is a haematological pathology that provokes an increase in blood viscosity and vascular resistance conferring a high risk of thrombosis and cardiovascular diseases. Finally, CMS renders ascents very susceptible to

myocardial infarction and stroke due to both higher blood viscosity and obstruction of blood supply to heart and brain, respectively [205].

Humans adapted to live at high altitude

Since the exposure to hypoxia leads to many physiological changes that can be dangerous for the human health, how is it possible that three populations, Tibetans, Andeans and Ethiopians could reside for millennia in a permanent hypoxic environment? The explanation can be found in the fact that hypoxia applied a selective pressure on these ethnic groups who overcame the low oxygen tension through unique physiological and genetic adaptations. Interestingly, these three populations have developed diverse adaptation mechanisms in terms of haematological response, pulmonary and cardiovascular changes as well as genetic modifications (Table 3). Tibetans show selectively low haemoglobin levels and low arterial oxygen saturation, whereas Andeans present higher haemoglobin concentrations and low oxygen saturation and Ethiopians display haemoglobin levels and oxygen saturation similar to sea-levels individuals [206]. Epo levels are also lower among Tibetans compared to Andeans reflecting the haemoglobin concentrations [207]. The typical hypoxic ventilator response (HVR) is not found in Andeans, who show no increase in resting ventilation [208], while Tibetans show sea-level HVR and higher resting rate compared to Asian and European populations acclimatized at the same altitude [209]. Particularly, Tibetans show a resting ventilation rate (19.7 L/min) 1.5 higher than Andeans [210]

Phenotype	**Andean**	**Tibetan**	**Ethiopian**
Resting ventilation	No increase	50% higher	NR
Hypoxic ventilatory response	Low	~ Sea-level value	NR
Arterial O_2 saturation	Elevated	No increase	Elevated
Haemoglobin concentration	Elevated	Minimal increase	Minimal increase
Pulmonary arterial pressure	Elevated	Minimal increase	Elevated
Nitric oxide	Elevated	Elevated	NR
Birth weight	Elevated	Elevated	NR

Table 3. Physiological adaptation to hypoxia among high altitude populations. NR: not reported. From [211].

As mentioned above, chronic pulmonary vasoconstriction is a common effect after hypoxia exposure and is present among Andeans likely due to the thickening of the pulmonary arterial walls [212], whereas Tibetans display only a minimal increase in HVR [213]. Furthermore, the vasodilator nitric oxide (NO) plays an important role in the adaptation to

high altitude as it regulates blood flow and vascular resistance. Tibetans exhibit high levels of NO accompanied by increased blood flow but not vascular resistance, suggesting that the higher concentration of NO might counteract hypoxic vasocontriction. Surprisingly, despite elevated arterial oxygen saturation and pulmonary arterial pressure in Andeans, the levels of NO are also augmented. High altitude has serious effects on pregnancy and fetal growth and can cause intrauterine growth restriction (IUGR), leading to low birth weight. However, newborn Tibetans and Andeans appear to be protected from birth weight reduction [214].

The distinct phenotypes described above for Tibetans, Andeans and Ethiopians suggest that differences in the adaptation to hypoxia occurred between these populations including modifications in specific genes and pathways [205]. Comparative genomic studies among these three populations analysed primarily the genes involved in the HIF pathway to screen for target genes for natural selection. Two SNPs within the HIF target gene *EGLN1*, encoding for the enzyme PHD2 which hydroxylases HIF, indicate that the lower hydroxylation of HIF leads to increased activation of the HIF pathway and consequent transcription of target genes contributing to the phenotypic adaptation of Tibetans [215]. Moreover, genome-wide studies identified also the gene *EPAS1* encoding for HIF-2α as candidate that underwent the hypoxia pressure in Tibetans. Curiously, it has been postulated that *EPAS1* may have a Denisovan origin, deriving from an extinct species of *Homo* found in Denisova cave (Siberia) [216]. Among Andeans, target genes involved in high altitude adaptation include variants in *EGLN1, PRKAA1,* linked to fetal growth and birth weight as well as *SENP1,* an enzymatic regulator of erythropoiesis that when down regulated ameliorates hypoxia tolerance [217]. Genomic scans performed among Ethiopians revealed genes involved in the adaptation to hypoxia belonging either to the HIF pathway such as ARNT2 and THRB or non-hypoxia-related genes playing a role in pathogen defence, angiogenesis or calcium uptake [218].

In conclusion, Andeans, Tibetan and Ethiopians adapted to high altitude through different genes and specific genetic changes and pathways. The possibility to link the genotype to the phenotype of these highlander populations is a future perspective of evolutionary biology that aims not only to expand the knowledge about the genetic variations under hypoxia selection but also to apply such information to discover the genetic elements causing chronic ischemic diseases as target of potential treatment of CMS [201].

1.6.2 Cellular response to hypoxia: the PHD/FIH-HIF-pVHL pathway

All nucleated mammalian cells respond to hypoxia by stabilizing the hypoxia–inducible factors (HIFs), the master regulators of oxygen homeostasis, leading to the activation of hundreds of genes involved in several cellular processes which need to adapt to oxygen deprivation such as glucose metabolism, angiogenesis, erythropoiesis, iron uptake and cell proliferation [219]. The HIF pathway is highly conserved across species, found in all metazoans and even in more ancient organisms like nematodes and corals [220]. Such evolutionary conservation might be explained by the fact that the HIF pathway plays a key role in the response to hypoxia at both cellular and systemic levels. An example is given by the enhanced transcriptional activation of the cytochrome oxidase subunits COX4-1 and COX4-2 that ameliorates the mitochondrial respiration at low O_2 concentration [221]. Similarly, the switch from aerobic to anaerobic glycolysis due to decreased oxygen availability triggers the upregulation of either the glucose transporters Glut-1 and Glut-4 to optimize the glucose uptake and all glycolytic enzymes [200].

The discovery of HIFs came from DNA-protein interaction studies at the downstream enhancer of the erythropoietin gene, the 3' hypoxia response element (3' HRE) in Hep3B cells in the laboratory of Gregg Semenza [222]. Successively, the characterization of the purified hypoxia inducible factor revealed that HIF is a heterodimer composed by a hypoxia-inducible α subunit of ~120 kDa and a constitutive β subunit of 91-94 kDa [223]. The HIF-α subunits comprise HIF-1α, HIF-2α or HIF-3α, which all present a similar basic protein structure (Figure 17). Starting from the N-terminus, the protein structure comprises: a helix-loop-helix (HLH) domain involved in DNA binding, followed by a two Per-Arnt-Sim (PAS) domains, responsible for protein-protein interaction with the HIF-β subunit, the oxygen-dependent degradation domain (ODDD) important for the HIF protein stability and the N- and C- terminal activation domains (NAD and CAD), conferring transcriptional activity. Finally, an inhibitory domain (ID) mediates normoxic repression of the CAD (Figure 17). The ODDD domain is missing in HIF-β, confirming its ubiquitous presence in normoxia [224]. While HIF-1α and HIF-2α heterodimerize with HIF-β to form an active transcriptional complex, HIF-3α shows weak transcriptional activity and is thought to be a negative regulator of the other two isoforms. In contrast to *HIF1A* and *HIF2A*, the human *HIF3A* locus is subjected to alternative splicing leading to the formation of several variants such as the inhibitory PAS domain protein (IPAS) that, after dimerization with HIF-β, inhibits target genes as well as the neonatal and embryonic PAS (NEPAS) also able to heterodimerize with HIF-β. However, due to its weak transcriptional activity, it is believed that HIF-3α might have a role in modulating the hypoxic response initiated by both HIF-1α and HIF-2α [225]. In addition to the HIFs,

another category of proteins has been discovered to possess a crucial function within the HIF pathway, which are termed prolyl-4-hydroxylase domain (PHD) enzymes. Such prolyl-4-hydroxylases are able to sense continuously O_2 levels due to their high Michaelis constant (K_m) for oxygen and low O_2 affinity and are, thus, considered as cellular oxygen sensors [133].

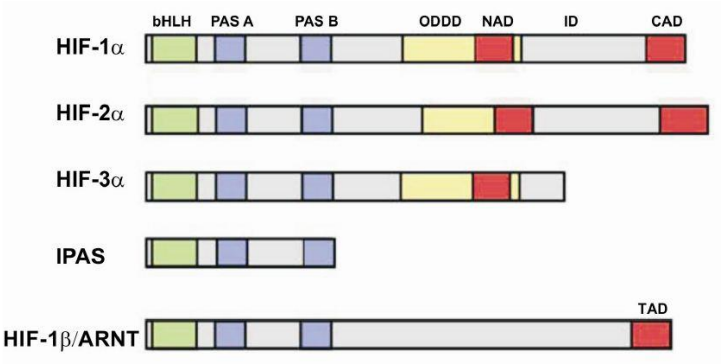

Figure 17. Structural domains of HIF-α, IPAS and HIF-β monomers. From [224].

Based on the sequence of the *egl-9* gene, which encodes a HIF regulating prolyl-hydroxylase in *C.elegans*, Ratcliffe and co-workers discovered the mammalian homologs HIF hydroxylase enzymes named PHD1, PHD2 and PHD3 [226]. The PHD enzymes exert their action on the ODD domain of HIFs. In the presence of sufficient oxygen (normoxia), PHDs hydroxylate two proline residues of HIF-α subunits Pro402 and Pro564 in human HIF-1α and Pro405 and Pro531 in human HIF-2α. This reaction requires O_2 and 2-oxoglutarate, converted to CO_2 and succinate, as well as ferrous iron and vitamin C. Hydroxylated HIF-α is then recognized by the von Hippel-Lindau tumour suppressor protein (pVHL), being part of an E3 ubiquitin ligase complex, and targeted for polyubiquitination and subsequent proteasomal degradation (Figure 18) [227] [219]. In conditions where oxygen demand exceeds oxygen supply (hypoxia), all the available oxygen is used up by the oxidative enzymes in mitochondria to fuel the oxidative phosphorylation. Subsequently, the insufficient oxygen levels prevent the hydroxylation of HIF-α which is stabilized, accumulates in the cytoplasm, translocates into the nucleus and binds to HIF-β to form a transcriptional active heterodimer (Figure 18) [133]. The HIF-α/HIF-β heterodimer recruits the co-factors CBP/p300 in order to regulate the transcription of specific target genes involved in the adaptation to hypoxia [228]. The CBP/p300 cofactor is a histone acetyltransferase and modulates the chromatin structure toward an “open” conformation favouring transcriptional activity [219]. Another member of the HIF hydroxylase enzymes is the Factor Inhibiting HIF (FIH), which hydroxylates HIF at an asparagine residue within the CAD of HIF-α: Asn803 in human HIF-1α and Asn851 in human HIF-2α. Asparaginyl hydroxylation does not prevent HIF-α stabilization but blocks the interaction of HIF-1α and HIF-2α subunits with the nuclear CBP/p300 coactivators. It is

believed that asparaginyl hydroxylation might provide a second oxygen-regulated mechanism by which the transcriptional activity of HIF-α is fine tuned [229]. PHD-mediated hydroxylation participates in the cross-talk between oxygen sensing and mitochondrial metabolism. Intermediates from the Krebs cycle such as malate/fumarate and succinate are inhibitors of PHDs as they compete with 2-oxoglutarate in binding the active site [230], while certain mutations in the gene encoding isocitrate dehydrogenase-1 (IDH-1), succinate dehydrogenase (SDH) and fumarate hydratase (FH) cause a reduction in 2-oxoglutarate levels increasing HIF-1α protein amounts [231] [232]. Additionally, PHD hydroxylase activity can be inhibited by divalent metal ions such as cobalt (Co^{2+}) or nickel (Ni^{2+}), which impede the entry of vitamin C into the cell as well as by reactive oxygen species (ROS) which, depending on dose and duration of exposure, oxydize Fe^{2+} to Fe^{3+} [232].

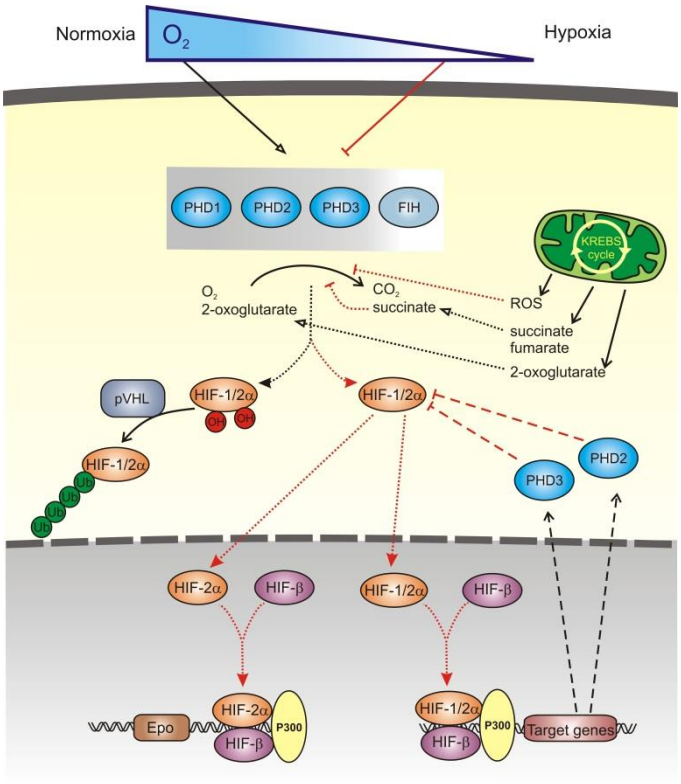

Figure 18. Overview of the PHD/FIH-HIF-pVHL pathway with its oxygen regulation and cross-talk with mitochondrial metabolism. From [133].

As shown in Figure 18, PHD2 and PHD3, but not PHD1 and FIH, are HIF target genes with hypoxic induction of PHD3 preceding PHD2 and both can, in turn, downregulate HIF-α proteins suggesting the existence of an intrinsic negative feedback mechanism [233] whose relevance for Epo regulation remains to be elucidated.

While HIF-1α mRNA is ubiquitously expressed in all nucleated cells of *Metazoans*, HIF-2α mRNA levels are particularly high in tissues relevant for the systemic delivery of O_2 like lung, heart and endothelium. In tissues expressing both HIF-α isoforms, the overlap of cellular

expression is variable. For example, hepatocytes, cardiomyocytes, and myocardial endothelial cells respond to hypoxia with up-regulation of both HIF-1α and HIF-2α, whereas in lung, kidney and brain the two subunits are found in different cell populations. Particularly, in the lung, HIF-2α protein is expressed in type II pneumocytes and pulmonary endothelial cells, while HIF-1α is not detectable; in the kidney HIF-1α is confined to renal tubuli, whereas HIF-2α is localized in peritubular interstitial cells, endothelial and glomerular cells [234] [235] and, in the brain, HIF-1α is expressed in neuronal cells and HIF-2α in glial cells [236].

The essential role played by the HIF pathway in the physiological adaptation to hypoxia is demonstrated by the fact that homozygous knock-out of either HIF-1α, HIF-2α, PHD2 or pVHL leads to embryonic lethality in mice [21] whereas embryos lacking PHD1 or PHD3 are viable [237]. Mice with homozygous deletion of *Hif-1α* die *in utero* between E8 and E11 due to neural tube defects and cardiovascular malformations [238]. Different phenotypes have been described for global *Hif-2α* knock-out: impaired catecholamine synthesis leading to heart failure and perinatal death [239], development of respiratory distress syndrome due to insufficient production of surfactant in the lung caused by deficient VEGF levels [240], mitochondrial abnormalities and deficiency in ROS homeostasis [241] and anaemia due to inadequate renal Epo production [242]. *Phd3*$^{-/-}$ mice show abnormal sympathoadrenal development and reduced systemic blood pressure [243], whereas *Phd1*$^{-/-}$ mice exhibited higher white adipose tissue (WAT) mass, despite lower body weight, hepatic steatosis and liver-specific insulin resistance [244]. In contrast to *Phd3*$^{-/-}$ and *Phd1*$^{-/-}$ mice, *Phd2*$^{-/-}$ mice display severe placental and heart defects followed by embryonic death. Moreover, two independent conditional PHD2 knock-out mouse models showed increased angiogenesis and erythropoiesis, demonstrating the dominant role of PHD2 in controlling renal Epo synthesis [245] [246] [247]. Mutations in *VHL* gene predispose to the development of a variety of tumours affecting most commonly retinal and central nervous system as well as kidney, leading to renal cancer of the clear cell phenotype [248]. In line with its central role in regulating the HIF pathway, specific mutations in the *VHL* gene provoke also erythrocytosis in mice [249] and humans as observed in Chuvash polycythaemia caused by a VHL R200W mutation in the absence of cancer [250].

The final step of the HIF pathway consists in the binding of HIF-α/β heterodimer at specific DNA element called hypoxia response element (HRE), present in the regulatory regions of hypoxia-regulated genes. The HIF DNA binding site within the core of the HRE is also termed HIF binding site (HBS) whereas HRE refers to the minimal *cis*-regulatory elements required to induce gene transcription upon hypoxia. Therefore, the HBS is the minimal DNA sequence required for interaction with HIF while a functional HRE contains the HBS as well as the neighbouring DNA binding sites for additional transcription factors (TFs), which are not

necessarily hypoxia inducible but their activity might amplify or block the hypoxia response and confer tissue-specific functions to the HRE [219] [251]. An example of interaction between HIF and TFs is the lactate dehydrogenase A gene, whose transcription is activated via cooperation of HIF, ATF-1 and CREB-1 at the HRE [252]. The analysis of validated HIF target genes has confirmed the consensus core HRE sequence to be 5'-RCGTG-3' (R= A or G), followed after 8 nucleotides by the sequence 5'-CACA-3' named the CACA repeat, to which no binding proteins have been identified yet. Intriguingly, regulatory regions of mammalian genes contain many more potential core HRE sequences compared to the ones effectively bound by HIF to regulate gene expression. How can HIFs discriminate between functional and non-functional HREs if the HRE core consensus sequence is identical? An explanation might be the presence of this 8 nucleotides sequence that is highly variable and could serve as DNA binding site of different TFs cooperating with HIFs, thus, conferring tissue and gene specificity [251]. An additional layer of complexity concerning the distinction of HREs by HIFs is found in epigenetic effects, which might contribute to reduce the number of functional HREs. In fact, the consensus core HRE contains CpG dinucleotide and its methylation would interfere with transcription factor binding to DNA via both direct steric hindrance and the binding of repressor proteins [253]. Surprisingly, two or three tandem core HREs are able to form a functional HRE as reported in genes encoding transferrin, several glycolytic enzymes and Glut-1 [251]. Using chromatin immunoprecipitation coupled to next-generation DNA sequencing (ChIP-seq), a recent report examined the pan-genomic patterns of HIF-1 and HIF-2 binding in hypoxic conditions and showed that, despite many HBS can bind both HIF isoforms, the binding distributions depend on the intrinsic properties of each isoform as *in vitro* disruption of either HIF-α subunit had a weak effect on the binding of the other. Moreover, when comparing the binding of HIF-1 and HIF-2, it has been observed that HIF-2α binds in a more cell-type specific manner and that HIF-2 binds more distantly (enhancers) from the transcriptional start site whereas HIF-1 binds more closely to the transcriptional start site (promoters). Taken together, the authors conclude that the factors determining the HIF-α isoform binding specificity might be different from those determining the cell-type specificity of HIF binding [254].

1.7 Transcriptional regulation of erythropoietin in hypoxia

1.7.1 Regulatory regions within the EPO locus

Erythropoietin is exclusively regulated on the mRNA level and, despite its important physiological functions and its great therapeutical potential, the molecular mechanisms behind Epo regulation is only partially elucidated. As mentioned before, Epo regulation is tissue-specific and developmental stage-specific, being the liver the major site of Epo production during fetal life and the kidney the main Epo producing organ in adulthood [111]. Moreover, Epo gene expression is induced by hypoxia [129], hypoxia-mimetics such as $CoCl_2$ [255] and by anaemia [256]. The *EPO* gene is localized as a single copy to the long arm of chromosome 7 in humans and to chromosome 5 in mice and it contains 5 exons and 4 introns [257]. Comparison between human and mouse *EPO* genes revealed sequence homology >75% [258]. Particularly, three noncoding segments of the EPO gene are highly conserved between human and mouse: the promoter, the first intron and a 120 bp region located at 100 bp downstream to the polyadenylation site [259]. The tissue specificity of Epo expression in liver and kidney might be explained by distinct *cis*-acting regulatory DNA elements at which different transcription factors bind, driving the activation of Epo transcription in a tissue specific manner. Such regulatory DNA elements modulating Epo expression might also be responsible for the switch between hepatic and renal Epo production since, from a developmental point of view, the liver arises from the embryonic endoderm and the kidney from the embryonic mesoderm [256]. The investigation of the *cis*-regulatory DNA elements controlling the tissue specificity as well as the hypoxia inducibility of Epo expression was accomplished by *in vivo* studies with transgenic mice carrying fragments of the human *EPO* gene encompassing different lengths of the 5' and 3' flanking regions as a renal cell line, able to produce Epo in a hypoxia-inducible manner, derived from the kidney was lacking. Semenza and collegues generated the first transgenic mouse (tgEPO4), which contained a 4 kb fragment containing human *EPO* gene, 0.4 kb upstream of the transcription start site (TSS) encompassing the *EPO* promoter, and 0.7 kb downstream of the polyA site (Figure 19A). Such transgenic mice were polycythaemic due to increased Epo mRNA levels in liver, kidney as well as other tissues. Moreover, hypoxia caused by anaemia induced Epo mRNA levels in liver, but not in kidney, suggesting the requirement of additional regulatory sequences for Epo induction in the kidney while the liver-inducible element (LIE) within the transgene construct conferred liver expression. The same group generated another transgenic mouse line (tgEPO10) that in addition to the 4 kb fragment included an expanded upstream region to the TSS of 6 kb resulting in a 10 kb final transgenic construct (Figure 19A).

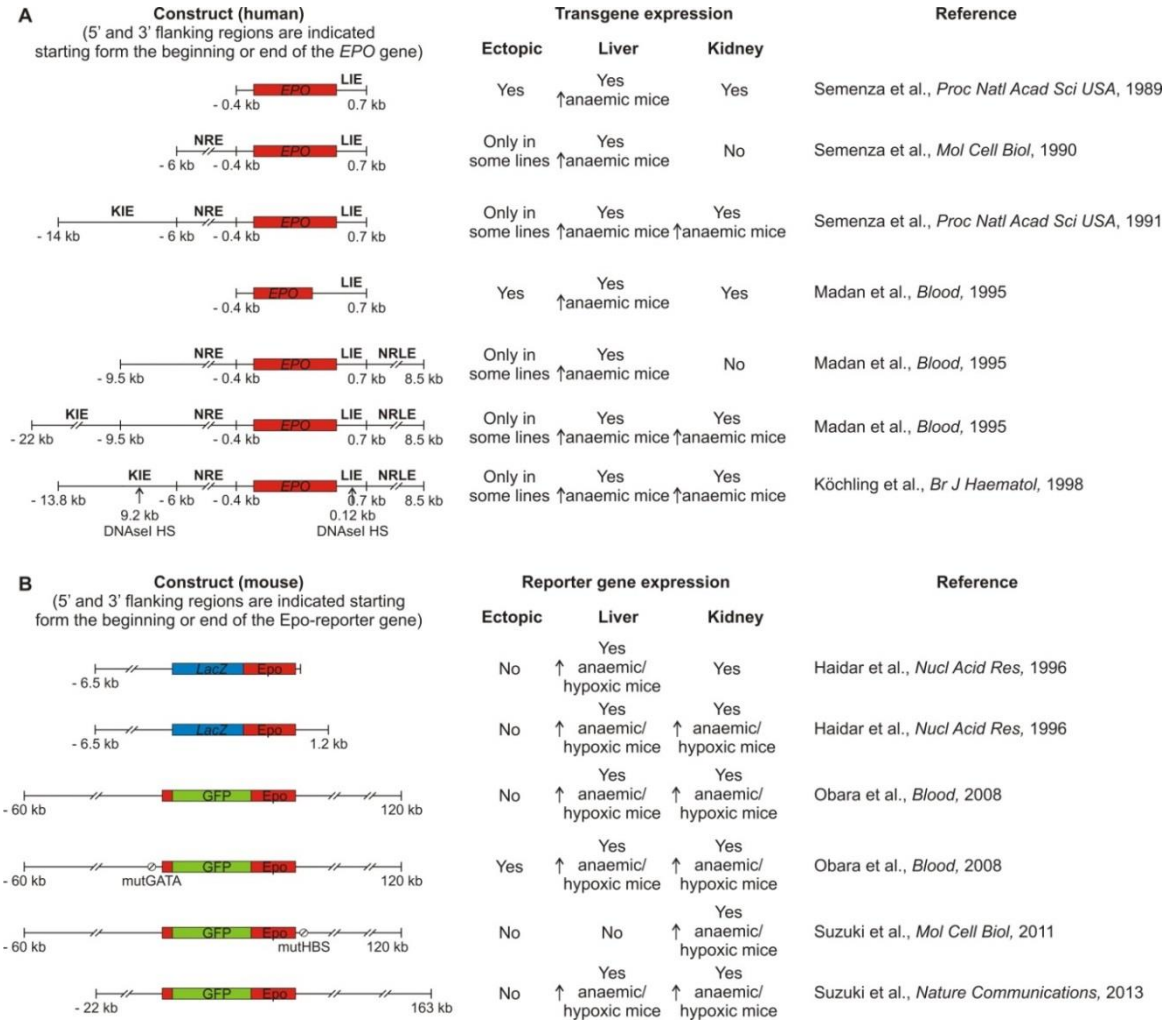

Figure 19. Overview of the human (A) and mouse (B) *EPO* gene constructs used to generate specific transgenic mice. Transgene expression pattern and references are displayed.

Similarly to the tgEPO4 mice, after inducing anaemia, these animals maintained increased Epo mRNA levels in liver but not in kidney. Therefore, none of tgEPO4 and tgEPO10 display regulated renal Epo expression, despite the kidney is the major site producing Epo in adulthood. In contrast to the tgEPO4 mice, the tgEPO10 mice displayed reduced Epo ectopic expression, implying that the sequence between -0.4 and -6 kb is a negative regulatory element (NRE), involved in suppressing Epo expression in tissues other than liver and kidney. Interestingly, in eukaryotic genes, NREs contribute to tissue-specific expression by suppressing constitutively the gene transcription in other tissues, whereas the tissue-specific induction relies on other positive regulatory elements such as enhancers [256]. In this regard, the *EPO* gene is an interesting example as it contains both negative and positive regulatory elements that function in concert to finely tune Epo expression in liver and kidney [256]. Successively, Semenza and colleagues further increased the size of the 5' flanking sequence to 14 kb, demonstrating that Epo gene expression was induced in both kidney and

liver in response to anaemia. This study demonstrated that the regulatory sequence responsible for modulating renal Epo induction, the so-called kidney inducible element (KIE) must be located between -14 and -6 from the TSS of the human *EPO* gene. Transgenic animals generated with this longer construct developed more severe polycythaemia than tgEPO4 and tgEPO10, probably due to Epo production in both kidney and liver [43]. To localize the sequences necessary for specific kidney Epo induction, Madan and collegues generated transgenic mice containing the same 10-kb fragment used by Semenza et al. along with additional 3' flanking sequence (7 kb from the polyA) and different 5' flanking sequences, including a more extended upstream region up to position -20 kb from the TSS containing Semenza's construct (-14 to -6 kb fragment of 8 kb) belonging to Epo33 mice or a smaller portion of Semenza's construct of 3.5 kb belonging to Epo22 mice. Epo22 mice showed inducible Epo mRNA levels due to anaemia exclusively in the liver, whereas Epo33 animals displayed Epo induction in both liver and kidney, confirming the findings of Semenza et al. and suggesting the localization of the KIE between -9.5 and -14 kb from TSS. Moreover, in contrast to the transgenic animals of Semenza et al., Epo22 and Epo33, having additional 7 kb of 3' flanking sequence, showed normal haematocrit and were only mildly polycythaemic, suggesting the presence of a negative regulatory liver element (NRLE) between 0.7 and 8.5 kb downstream of *EPO.* To verify whether introns might have a role in Epo regulation, Epo3 transgenic animals containing an *EPO* minigene lacking introns II-IV but including 0.4 upstream and 0.7 downstream sequences, were generated: Epo ectopic expression with liver but not kidney inducible expression was detected, indicating that such introns do not contain regulatory sequences responsible for Epo regulation or Epo induction in the liver [44]. Later, DNaseI hypersensitive site (HS) mapping in nuclei of kidney and liver of anaemic mice revealed the presence of open chromatin structure within the putative KIE and LIE proving their potential role in regulating inducible Epo [260] [261]. Successively, a new series of vectors containing reporter genes were used to study Epo regulatory elements. The LacZ reporter construct encoding β-galactosidase permitted to identify the presence of a liver-specific silencing element within 1.2 kb from the polyA of the *EPO* gene (Figure 19B) [262]. The best approach to study Epo regulation revealed to be the generation of bacterial artificial chromosome (BAC) transgenic mice expressing GFP as reporter gene under the control of a 180-kb inserted 60 kb upstream and 120 kb downstream of the mouse *Epo* gene. Interestingly, such construct fully recapitulated inducible physiological Epo expression in a tissue-specific manner allowing the characterization and isolation of renal Epo-producing (REP) cells [149]. Furthermore, mutated versions of this model were employed to assess the functionality of different transcription factor binding sites, such as the GATAs and HIF itself [263]. Successively, the group of Yamamoto generated other transgenic animals carrying a BAC construct encompassing 22 kb 5' to the TSS and 163 kb 3' to the polyA. This model

showed a GFP expression pattern representing the physiological Epo expression and was used to study embryonic Epo expression in neural crest cells [264].

1.7.2. Tissue-specific Epo regulation

The discovery of the two hepatoma cell lines Hep3B and HepG2, capable to produce Epo in a hypoxia-inducible manner has provided an invaluable tool in advancing the understanding of the molecular basis of *EPO* gene regulation and proved that even *in vitro* cells possess the necessary machinery for oxygen sensing and gene regulation [129]. The availability of such cellular models has allowed the characterization of the tissue-specific regulatory regions determined by transgenic animal studies. The discovery of HIF and its binding site was based on transient expression assay in Hep3B when Semenza and colleagues validated a 50-bp sequence within the LIE as hypoxia inducible enhancer [222]. In parallel, Madan et al. reported that in Hep3B a 621-bp fragment containing Epo promoter along with 5' flanking region was sufficient to drive a modest but statistically significant induction (2.4 fold) of luciferase activity in response to hypoxia. They also confirmed the presence of a HRE to a 24-bp portion of the 3' flanking region of *EPO* [265]. In line with these findings, Blanchard et al. identified a 117 bp fragment upstream of TSS containing the minimal human *EPO* promoter sequence required for hypoxic inducibility. Despite Epo promoter is *per se* a weak promoter (6-fold hypoxic induction), the combination of the *EPO* promoter and 3' enhancer increased the induction from 50- to 100-fold *in vitro*, demonstrating synergistic cooperativity between these transcriptional elements [266-268]. Intriguingly, DNA sequence comparison between the *EPO* promoter and the 3' enhancer revealed sequence similarities as the 10-bp sequence 5'-CACACAGCCT-3' is conserved. Interestingly, the *EPO* promoter contains twice the 10-bp sequence CACGCACACA [266], which contains a HIF binding site and will be further discussed as relevant for this thesis work. In the Epo promoter, while the typical TATA box is absent, a GATA box is present which is recognised and bound by GATA factors [269], belonging to the family of zinc finger DNA-binding proteins and playing a role in regulating haematopoiesis [270]. In hepatoma cells, two GATA sites have been identified that repress Epo transcription under normoxic conditions and are downregulated in hypoxia to ensure hypoxic Epo. As a consequence of the high homology between the human and mouse Epo genes, a GATA motif has been found also within the mouse Epo promoter region to which GATA-2 and GATA-3 factors bind and repress mouse Epo transcription [271]. Similarly, knock-down and over-expression experiments performed in hepatoma cells demonstrated that GATA-2 and GATA-3 have an inhibitory transcriptional effect on *EPO*, whereas GATA-4 revealed positive transcriptional effects on *EPO* gene [272] [273]. GATA-4 is highly expressed in fetal hepatocytes, whereas in the adult liver GATA-4 expression is low and

confined to epithelial cells surrounding the biliary ducts. Given its strong binding activity to the *EPO* promoter as well as its developmental stage specificity, it has been suggested that GATA-4 contributes to the switch of Epo gene expression from the fetal liver to the adult kidney [273]. Furthermore, the relevance of the GATA binding site has been demonstrated by *in vivo* studies where the GATA motif within the *EPO* promoter was disrupted in a GFP-reporter BAC construct: mutant animals displayed ectopic GFP expression in the epithelium of the lungs, kidneys and gallbladders, confirming the role of GATA factors in suppressing ectopic Epo expression by inhibiting its transcription [149]. A recent work showed that GATA factor inhibitors such as mitoxantrone and its analogues highly induced Epo expression in epithelial cells (normally non Epo-producing cells) derived from mouse and humans, independent of any hypoxic stimulus, further confirming the role of GATA factors as repressors of ectopic Epo expression and suggesting that mitoxantrone-mediated Epo induction occurs independently of the hypoxia response pathway [274].

Another factor named hypoxia-associated factor (HAF) has been identified to bind a 17 bp sequence (-61 to -45 bp) within the minimal Epo promoter (117 bp). Interestingly, HAF antisense experiments in Hep3B showed reduced Epo expression in hypoxia without abrogating the basal level, indicating that HAF synergizes with other TFs to modulate oxygen-regulated Epo transcription [275].

The Wilms tumour suppressor Wt1 is a transcription factor identified in paediatric renal carcinomas due to its inactivation and it has been suggested to be involved in Epo regulation for the following reasons: *(I)* The *EPO* promoter contains several predicted binding motifs for Wt1 (GnGGGnGnG) that are highly conserved in mice and humans; *(II) Wt-1* deficient mice display defects in the haematopoietic system and *Wt1*$^{-/-}$ embryos show reduced Epo expression in the liver, linking Wt-1 with Epo regulation; *(III)* Wt1 developmental expression is confined in a spatial and temporal manner like Epo expression. Particularly, Wt1 encodes a zinc finger protein presenting 4 alternative Wt1 splice variants, among which the insertion of the lysine-threonine-serine (KTS) tripeptide into the zinc finger domain reduces the DNA binding affinity of Wt1 (Wt1(+KTS)), whereas the lack of KTS increases the DNA binding affinity of Wt1 (Wt1(-KTS)) [276]. Dame et al. reported that Wt1(-KTS) augmented Epo promoter activity by 25-fold in human osteosarcoma U2OS cells, 9-fold in human embryonic kidney HEK293 cells and 5-fold in HepG2 cells in reporter gene assays under normoxic conditions. Moreover, since Wt1 does not up-regulate Epo in response to hypoxia, the authors suggested that Wt1(-KTS), in addition to GATA-4, is a transcriptional activator of basal Epo expression in the fetal liver and might be involved in the switch of Epo synthesis from the liver to the kidney [277].

In vitro studies in Hep3B and HepG2 cells allowed the molecular characterization of the 3' enhancer within the LIE and three important sites have been found to be involved in the response to hypoxia: *(I)* an HRE consensus sequence 5'-CACGTGCT-3' located at the 3'-end containing the HIF binding site (underlined); *(II)* a highly conserved CACA repeat located 7 bp after the HRE; *(III)* a direct repeat 2 (DR-2) site containing a binding site for hepatocyte nuclear factor-4α (HNF4α) consisting in a direct repeat of two steroid receptor half sites separated by 2 bp. As afore mentioned, this HRE within the 3' enhancer, also termed 3' HRE, is responsible for hypoxic induction of Epo mRNA by enhancing the minimal *EPO* promoter activity and has been demonstrated to be bound by HIF. In addition to bind the HBS, HIF binds also the constitutive coactivators p300/cAMP response element-binding protein (CREB)-binding protein (CBP), which are thought to indirectly interact with the promoter and the 3' HRE in order to enhance Epo transcription [278] [222] [266] [268].

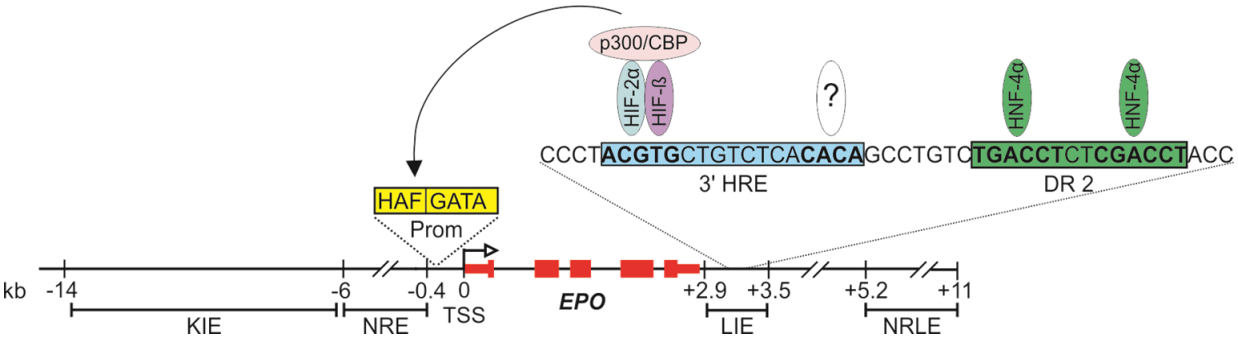

Figure 20. Scheme depicting the human *EPO* locus and its hypoxia response elements in liver and hepatoma cells. The *EPO* promoter is represented with a yellow box including the hypoxia-associated factor (HAF) and GATA binding sites. The light blue box within the LIE represents the 3' HRE region including the HBS (bold) bound by HIFs and the CACA repeat (bold) to which no binding factors have been identified yet. HIFs recruit p300/CBP co-activators at *EPO* promoter in order to enhance Epo transcription. The green box within the LIE represents the DR-2 sites to which HNF-4α bind ensuring hepatic specific Epo expression. Numbers indicate the distance in kb from the TSS (-: upstream, +: downstream). Adapted from [279].

Although no factor binding to the CACA repeat *(II)* has been identified yet, this element is known to be necessary but not sufficient to induce Epo transcription in hypoxia, acting as an ancillary sequence to the HBS [222]. The DR-2 site *(III)* has been reported to bind non-oxygen regulated protein complexes and its mutation markedly inhibited Epo transcription in hypoxia, confirming its involvement in hepatic Epo regulation. Several proteins are able to bind the DR2 site, among which HNF-4α has been detected [266]. HNF-4α is expressed in the renal cortex and liver like Epo and since its mutation abrogated Epo induction in Hep3B cells, it is believed that it may contribute to the hepatic Epo expression [280]. *In vivo* studies performed by the group of Volker Haase convincingly demonstrated that HIF-2α, but not HIF-1α, preferentially regulates hepatic Epo expression in both *Vhlh*-deficient and WT anemic mice as well as infant livers before switching Epo production to the kidney. By Chromatin

Immunoprecipitation (ChIP) analysis in Hep3B, the authors confirmed that also *in cellulo* HIF-2α preferentially associated with EPO 3' HRE in hypoxia and conclude that HIF-2α is the HIF isoform that mainly binds to the 3' enhancer within the LIE [281]. Importantly, 3 bp mutation of the 3' HRE in Epo-GFP reporter transgenic mice carrying a BAC construct caused abrogation of hypoxia-induced Epo expression in the liver, but not in the kidney with consequent anemia at late embryonic and neonatal stages due to defective hepatic erythropoiesis. Interestingly, the mutant mice were able to recover from the severe anemia during childhood when Epo production switched from the liver to the kidney [282]. This work validated that the 3' HRE is necessary and sufficient for both the liver-specific and hypoxia-responsive Epo expression after E14.5 and demonstrated that the 3' HRE is dispensable for Epo regulation in the kidney, suggesting the involvement of an additional HRE regulating renal Epo expression [282]. Intriguingly, a putative HRE consensus sequence, including the HBS and the CACA repeat, homologous to the HRE sequence of the well-known 3' HRE within the LIE has been mapped by a DNase I hypersensitivity assays in nuclei of anemic kidneys at -9.2 kb from the TSS within the KIE. These findings support the hypothesis that an additional regulatory element must be present within the KIE to control hypoxia-inducible Epo gene expression in the kidney [261]. The first study performing a functional characterization of this novel HRE, termed 5' HRE was executed by our group. We could, indeed, verify that the 5' HRE mapped to 9248 bp upstream from the TSS and that includes the HBS 3'-CACGT-5' (reverse strand) followed after 8 bp by the CACA repeat, in a very similar way to the established 3' HRE. Furthermore, we confirmed that this putative 5' HRE is strongly conserved in multiple vertebrate species and its sequence overlaps with the same DNase I hypersensitive-site as mentioned above. Moreover, we could show that the 5' HRE is functionally active in hypoxia in both Epo-producing and non Epo-producing cells by reporter gene assays and observed hypoxia-induced HIF-2α enrichment at the 5' HRE whereas both HIF isoform were found to bind the 3' HRE [279].

The current knowledge on Epo regulation is summarized in Figure 21. In case of limited oxygen supply, HIF-α isoforms are stabilized and translocate to the nucleus where they bind HIF-β at the HBS within the 3' HRE and recruit p300/CBP coactivators and HNF-4α at the DR2 site. Upon binding, the HIFs/p300/CBP/HNF-4α complex interacts with the transcription factors bound at the *EPO* promoter (e.g. GATA-4) by "bridging" the 3' HRE to the promoter element (Figure 21), enhancing Epo transcription in hypoxia up to 100-fold. While the oxygen-regulated functionality of the 5' HRE has been shown through our previous reporter assays [279], its endogenous activity and role in Epo regulation in the kidney remains to be validated. Due to the lack of a renal cell line able to induce Epo expression in a hypoxia-inducible manner, the endogenous characterization of the novel 5' HRE cannot be executed

within the relevant renal context. However, the evaluation of its endogenous relevance and its role in relation to the well-established 3' HRE and *EPO* promoter could be carried out in other Epo-producing cell lines originating from organs known to produce Epo during hypoxia like the brain. Especially, the neuroblastoma cell line Kelly, derived from sympathetic neuroblasts of the peripheral nervous system, has been reported to express Epo in an oxygen-dependent and HNF-4α independent manner, and has thus been proposed as a new *in vitro* system to study hypoxia-inducible Epo expression in human neuron-like cells [283].

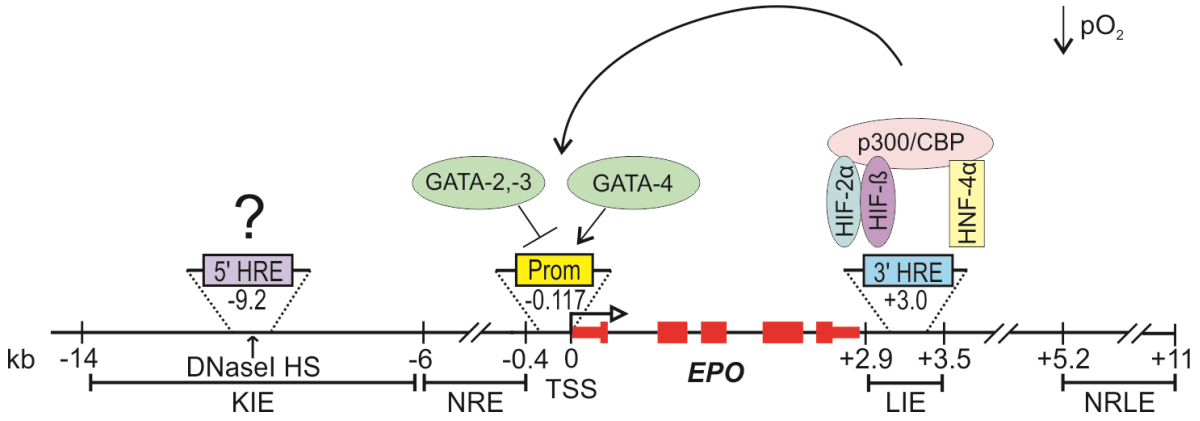

Figure 21. Scheme displaying oxygen-regulated hepatic Epo transcriptional regulation. The violet squared box represents the novel 5' HRE localized at -9.2 kb upstream the TSS in correspondence of a DNaseI HS and its endogenous role in regulating Epo in the kidney is a matter of debate. The preferential HIF-α isoform binding the liver-specific 3' HRE is HIF-2α as previously demonstrated *in vivo* and *in vitro* [281]. From [279].

An additional layer of complexity regarding Epo regulation is the potential role that methylation might have on the *EPO* locus. Methylation of CpG sites within *EPO* promoter represses Epo expression as methyl CpG-binding proteins are recruited and interfere with other TFs or interact with histone deacetylases and corepressors. Moreover, it has been demonstrated that in Epo-producing cells the CpG site at Epo promoter are less methylated compared to non Epo-producing cells and the methylation status seemed to be partly hypoxia-regulated [284]. Methylation of the HBS within the 3' HRE prevents HIF binding and inversely correlated with Epo expression in cell lines and tissues [253] [283]. Therefore, it seems that both a methylation-free HBS and hypoxic conditions are necessary to have HIF binding at the 3' HRE followed by Epo induction. Interestingly, treatment of several cancer cell lines with the DNA methylation inhibitor 5'-aza-2'-deoxycytidine (Aza) restored hypoxic Epo expression in neuroblastoma but not in Hep3B cells where likely the *EPO* locus is de-methylated having then the chromatin in an "open status" ready to be transcribed [285]. In line with the results obtained in neuroblastoma cells, a recent work reported that Epo expression in kidney myofibroblast was recovered after treatment with low nontoxic doses of Aza, indicating a potential role of demethylating drugs in Epo regulation and anemia [286].

Finally, methylation affects tissue-specific Epo expression by repressing or silencing the *EPO* locus and is thus an important aspect of Epo regulation that deserves further investigation.

1.7.3 HIF/PHD isoforms regulating Epo

In situ hybridization studies performed by Rosenberger et al. analyzed the expression pattern of HIF-1α and HIF-2α in hypoxic rat kidneys, demonstrating that HIF-2α isoform is the only isoform localized in Epo producing peritubular fibroblasts. This finding demonstrated a role for HIF-2α in renal Epo regulation in line with *in vivo* studies where postnatal global removal of HIF-2α but not HIF-1α, resulted in anemia treatable with recombinant Epo. Further evidence supporting a role of HIF-2α in Epo regulation came from hepatic HIF-2α knock-out mouse models developing postnatal anemia that resolved in adulthood when the kidney replaced the liver in producing Epo; overexpression of HIF-2α but not HIF-1α led to erythrocytosis. Also a liver-specific VHL knock-out resulted in polycythemia that was rescued only by deleting HIF-2α but not HIF-1α. Moreover, by RNA interference, Warnecke et al. down-regulated HIF-1α or HIF-2α in Hep3B and Kelly cells and observed a significant decrease in Epo levels only when HIF-2α but not HIF-1α was silenced, confirming that Epo is a HIF-2α target gene in both hepatoma and neuroblastoma cell lines. Interestingly, mutations identified in human *EPAS1* cause hereditary erythrocytosis while no mutations in *HIF1A* or *HIF3A* causing a similar phenotype have been reported.

The cross-talk between iron metabolism and HIF-2α, demonstrated by the fact that genes involved in iron metabolism such as DMT-1 (divalent metal transporter 1) and DcytB (duodenal cytochrome b) are HIF-2α target genes, supports a major role of HIF-2α in Epo regulation. Genome wide association studies (GWAS) comparing lowlanders to highlanders found polymorphisms in the *EPAS1* and *EGLN1* associated with RBC mass, validating the association of HIF-2α as well as PHD2 with Epo regulation. In fact, among the PHD isoforms, inducible knock-out models only for PHD2 showed increased Epo levels, polycythemia and angiogenesis, indicating PHD2 as the enzyme that predominantly regulating HIF-2α stability upstream of Epo transcription. Finally, mutations in the gene encoding for PHD2 (*EGLN1*) but not in the gene encoding for PHD1 *(EGLN2*) or PHD3 (*EGLN3*) have been identified in families affected by congenital erythrocytosis, further confirming the crucial role of PHD2 in regulating Epo expression. Taken together, the above findings suggest that pVHL/HIF-2α/PHD2 is the main axis regulating Epo production during hypoxia.

1.8 Pathophysiology of Epo

The physiological regulation of erythropoietin is crucial to maintain correct Epo levels within the body. In fact, the dysregulation of Epo production causes pathological conditions such as erythrocytosis (or polycythemia) and anemia. While erythrocytosis occurs in case of Epo overproduction, anemia occurs when Epo is insufficiently produced. Besides erythrocytosis and anemia, injuries or medical procedures damaging the kidney can impair Epo production. An example is the kidney allotransplantation, which has been used in earlier times to treat anemia by restoring Epo production and, then, replaced by rhEpo treatment [21].

Erythrocytosis can be classified as primary, if the defects are related to the hematopoietic progenitors leading to excessive RBC synthesis, or secondary, when the impairment of Epo production causes increased RBC synthesis. Erythrocytosis can be acquired or congenital [287]. The most common form is called *polycythemia vera*, a primary acquired disease, whereas the rarest is a primary congenital form due to mutations in EpoR resulting in constant activation of the erythropoietic pathway that leads to exaggerated RBC formation [288]. Conditions that cause an increase in Epo levels lead to acquired secondary erythrocytosis: use of Epo-stimulating agents, tissue hypoxia due to different aetiology (e.g. cardiovascular disease, tumor), exposure to high altitude, chronic lung disease, carbon monoxide poisoning, smoking and kidney transplantation [289]. Secondary congenital erythrocytosis is instead due to inherited mutations in genes involved in oxygen sensing and transport. Mutations altering the bisphosphoglycerate mutase synthetizing 2,3-DPG, the haemoglobin gene or the pVHL/HIF-2α/PHD2 axis influencing EPO transcription have been reported in families affected by erythrocytosis [290]. The main symptoms of erythrocytosis include increased blood viscosity caused by high RBC mass causing fatigue, abdominal pain, headache and blurred vision. High blood viscosity raises the risk for thrombosis. The therapy against thrombosis consists in venesection to remove surgically the excessive RBC mass from the vein or the administration of anti-coagulants like aspirin, depending on the clinical case [289]. Curiously, homozygous R200W mutation in *VHL* causes an autosomal recessive form of polycythemia, termed “Chuvash polycythemia” so called as found for the first time in the Russian region Chuvash. Such mutation impairs the binding of pVHL to hydroxylated HIF-α isoforms, enhancing HIF stabilization. Surprisingly, despite having a defective *VHL*, the Chuvash polycythemia does not lead to cancer malignancy [291].

Interestingly, a missense mutation (G269A substitution) in the *BPGM* gene encoding for bisphosphoglycerate mutase (responsible for the synthesis of 2,3-BPG from 1,3-BPG) has been reported to cause erythrocytosis by reducing the intracellular levels of 2,3 BPG in RBC,

resulting in a leftward shift in the hemoglobin-oxygen dissociation curve and increase in O_2 affinity. [292].

Anemia is the most common haematological disorder due to reduced haemoglobin content of the blood (Hb < 13g/dL in men and <12g/dL in women). Anemia can be caused by several defects which are mainly grouped in two main categories: the amount of newly produced erythrocytes is inadequate to meet the oxygen demand of the tissues or the number of RBC is sufficient but the erythrocytes are not functional (e.g. sickle-cell anemia). Therefore, the primary symptom is insufficient delivery of oxygen to the periphery leading to pallor, fatigue and dyspnea. Anemia can be provoked as secondary pathology by nutritional deficiencies (iron or vitamin B_{12}), acute or chronic blood loss, haemoglobinopathy, caused by mutations within the Hb-gene rendering oxygen transport inefficient, bone marrow failure and renal failure [293].

Renal failure occurring for example in chronic kidney disease (CKD) leads to impaired Epo synthesis due to destruction of the renal architecture. CKD is a pathological condition caused by genetic and environmental factors such as hypertension, diabetes, age, obesity and smoking that increase the cardiovascular risk and ultimately renal failure. In fact, high blood pressure or toxic substances challenge the kidney in filtering the blood damaging the nephrons. In addition, infections or auto-immune disease can provoke kidney injury due to inflammation and fibrosis that eventually progresses to CKD. Renal damage is diagnosed by decreased glomerular filtration rate (GFR) and proteinuria. Patients at the last stage of CKD need dialysis, hemofiltration or transplantation to survive. Anemia is a complication of CKD that arises from renal dysfunction causing incorrect Epo production and erythropoiesis [294]. Consistently, Epo levels are blunted and do not respond appropriately to the anemic stimulus [295]. However, CKD patients are still able to produce Epo when exposed to hypoxia [296], while anemic patients require less doses of rhEpo when residing at high altitude as they respond more efficiently to the rhEpo treatment, probably due to increased endogenous Epo production [297]. The reason why Epo production is inappropriately low in diseased kidneys remains not fully understood. The current hypothesis suggests that during renal failure oxygen consumption decreases causing higher tissue pO_2 that impede REP cells to reach the hypoxic threshold required to induce Epo [133]. Of note, in CKD inflammatory cytokines such as TNFα, known to inhibit Epo synthesis [298], trigger the transformation of REP cells to myofibroblasts, which loose the capability of producing Epo and, therefore, are considered the link between fibrosis and anemia. Intriguingly, a short-term reversible UUO model demonstrates that transformed myofibroblasts re-acquire after 12 days their physiological morphology and ability to synthetize Epo, suggesting that REP cells have high cellular

plasticity, which awaits further molecular characterization but opens new possibilities in treating CKD by protecting or regenerating REP cells via epigenetical modifications [152].

Finally, given the recent insights into the oxygen sensing mechanism, a new strategy in treating CKD based on small and cheap molecules analogous of 2-oxoglutarate has been developed. Such compounds, termed PHD inhibitors, compete with 2-oxoglutarate in binding the active site of the PHD enzymes, leading to increased activation of the HIF pathway and Epo transcription. Because the HIF pathway regulates the transcription of hundreds of genes, it is crucial to target specifically the PHD isoform that hydroxylates distinct HIF-α subunits regulating the gene of interest to avoid pleiotropic effects. As mentioned above, PHD2 is the PHD isoform that predominantly hydroxylates HIF-2α which drives the transcriptional activation of erythropoietin in both adult liver and kidney [299]. Therefore, the actual approach to treat renal anemia is the use of PHD2 inhibitors that showed efficient effects *in vitro* and proved to be beneficial *in vivo* as they ameliorated several diseases such as tissue ischemia, kidney injury and transplantation [300] [301]. The most advanced clinical studies (Phase III trials) use roxadustat (FG-4592) to correct renal anemia. Moreover, roxadustat reduces the hepcidin levels leading to higher iron availability as iron export (from the gut to the hepatic portal system), normally inhibited by hepcidin, becomes less constrained, and iron absorption increases to sustain RBC formation. Currently, clinical studies are examining the long-term cardiovascular outcomes and possible side effects of roxadustat [302]. In conclusion, despite roxadustat seems to be a promising drug for the treatment of renal anemia in CKD, its molecular mechanism and the repercussions of HIF-2α stabilization on other HIF-2α target genes need further investigations to figure out the risks associated with this new therapy.

1.9 References

1. Jelkmann, W., *Regulation of erythropoietin production.* J Physiol, 2011. **589**(Pt 6): p. 1251-8.
2. Jelkmann, W., *Functional Significance of Erythrocytes.* Erythrocytes, 2012.
3. Jelkmann, W., *Erythropoietin research, 80 years after the initial studies by Carnot and Deflandre.* Respir Physiol, 1986. **63**(3): p. 257-66.
4. Erslev, A.J., *Physiologic control of red cell production.* Blood, 1955. **10**(9): p. 954-61.
5. Reissmann, K.R., *Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia.* Blood, 1950. **5**(4): p. 372-80.
6. Jacobson, L.O., et al., *Role of the kidney in erythropoiesis.* Nature, 1957. **179**(4560): p. 633-4.
7. Nathan, D.G., et al., *Erythropoiesis in Anephric Man.* J Clin Invest, 1964. **43**: p. 2158-65.
8. Bunn, H.F., *Erythropoietin.* Cold Spring Harb Perspect Med, 2013. **3**(3): p. a011619.
9. Goldwasser, E., *Erythropoietin: a somewhat personal history.* Perspect Biol Med, 1996. **40**(1): p. 18-32.
10. Jacobs, K., et al., *Isolation and characterization of genomic and cDNA clones of human erythropoietin.* Nature, 1985. **313**(6005): p. 806-10.
11. Lin, F.K., et al., *Cloning and expression of the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1985. **82**(22): p. 7580-4.
12. Winearls, C.G., et al., *Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis.* Lancet, 1986. **2**(8517): p. 1175-8.
13. Eschbach, J.W., et al., *Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial.* N Engl J Med, 1987. **316**(2): p. 73-8.
14. Jelkmann, W., *Developments in the therapeutic use of erythropoiesis stimulating agents.* Br J Haematol, 2008. **141**(3): p. 287-97.
15. Jelkmann, W., *Recombinant EPO production--points the nephrologist should know.* Nephrol Dial Transplant, 2007. **22**(10): p. 2749-53.
16. Gupta, N. and J.B. Wish, *Erythropoietin mimetic peptides and erythropoietin fusion proteins for treating anemia of chronic kidney disease.* Curr Opin Nephrol Hypertens, 2018. **27**(5): p. 345-350.
17. Robinson, N., et al., *Erythropoietin and blood doping.* Br J Sports Med, 2006. **40** Suppl 1: p. i30-4.

18. John, M.J., et al., *Erythropoietin use and abuse.* Indian J Endocrinol Metab, 2012. **16**(2): p. 220-7.
19. Rafael Maia de Almeida Bento, L.M.P.D.a.F.R.d.A.N., *Recombinant human erythropoietin in sports: a review.* Rev Bras Med Esport, 2003.
20. Beguin, Y., *Soluble transferrin receptor for the evaluation of erythropoiesis and iron status.* Clin Chim Acta, 2003. **329**(1-2): p. 9-22.
21. Wenger, R.H. and A. Kurtz, *Erythropoietin.* Compr Physiol, 2011. **1**(4): p. 1759-94.
22. Schuler, B., et al., *Optimal hematocrit for maximal exercise performance in acute and chronic erythropoietin-treated mice.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 419-23.
23. Juvonen, E., et al., *Autosomal dominant erythrocytosis caused by increased sensitivity to erythropoietin.* Blood, 1991. **78**(11): p. 3066-9.
24. Fried, W., *The liver as a source of extrarenal erythropoietin production.* Blood, 1972. **40**(5): p. 671-7.
25. Zanjani, E.D., et al., *Studies on the liver to kidney switch of erythropoietin production.* J Clin Invest, 1981. **67**(4): p. 1183-8.
26. Murphy, W.G., *The sex difference in haemoglobin levels in adults - mechanisms, causes, and consequences.* Blood Rev, 2014. **28**(2): p. 41-7.
27. Koury, M.J. and V.H. Haase, *Anaemia in kidney disease: harnessing hypoxia responses for therapy.* Nat Rev Nephrol, 2015. **11**(7): p. 394-410.
28. Geiduschek, J.B. and S.J. Singer, *Molecular changes in the membranes of mouse erythroid cells accompanying differentiation.* Cell, 1979. **16**(1): p. 149-63.
29. McGrath, K.E., et al., *Enucleation of primitive erythroid cells generates a transient population of "pyrenocytes" in the mammalian fetus.* Blood, 2008. **111**(4): p. 2409-17.
30. Palis, J., et al., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. **126**(22): p. 5073-84.
31. Stamatoyannopoulos, G., *Control of globin gene expression during development and erythroid differentiation.* Exp Hematol, 2005. **33**(3): p. 259-71.
32. Yi, Z., et al., *Sox6 directly silences epsilon globin expression in definitive erythropoiesis.* PLoS Genet, 2006. **2**(2): p. e14.
33. Kingsley, P.D., et al., *Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis.* Blood, 2004. **104**(1): p. 19-25.
34. Malik, J., et al., *Erythropoietin critically regulates the terminal maturation of murine and human primitive erythroblasts.* Haematologica, 2013. **98**(11): p. 1778-87.
35. Boussios, T., J.F. Bertles, and E. Goldwasser, *Erythropoietin. Receptor characteristics during the ontogeny of hamster yolk sac erythroid cells.* J Biol Chem, 1989. **264**(27): p. 16017-21.

36. McGann, J.K., et al., *Erythropoietin-receptor expression and function during the initiation of murine yolk sac erythropoiesis.* Exp Hematol, 1997. **25**(11): p. 1149-57.
37. Lin, C.S., et al., *Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis.* Genes Dev, 1996. **10**(2): p. 154-64.
38. Wu, H., et al., *Inactivation of erythropoietin leads to defects in cardiac morphogenesis.* Development, 1999. **126**(16): p. 3597-605.
39. Suzuki, N., et al., *Erythropoietin production in neuroepithelial and neural crest cells during primitive erythropoiesis.* Nat Commun, 2013. **4**: p. 2902.
40. Wu, H., *Generation of Committed Erythroid BFU-E and CFU-E Progenitors Does Not Require Erythropoietin or the Erythropoietin Receptor.* Cell press, 1995.
41. Koury, M.J. and M.C. Bondurant, *Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells.* Science, 1990. **248**(4953): p. 378-81.
42. Semenza, G.L., et al., *Polycythemia in transgenic mice expressing the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1989. **86**(7): p. 2301-5.
43. Semenza, G.L., et al., *Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice.* Proc Natl Acad Sci U S A, 1991. **88**(19): p. 8725-9.
44. Madan, A., et al., *Regulated basal, inducible, and tissue-specific human erythropoietin gene expression in transgenic mice requires multiple cis DNA sequences.* Blood, 1995. **85**(10): p. 2735-41.
45. Grote Beverborg, N., et al., *Erythropoietin in the general population: reference ranges and clinical, biochemical and genetic correlates.* PLoS One, 2015. **10**(4): p. e0125215.
46. Cotes, P.M. and B. Brozovic, *Diurnal variation of serum immunomoreactive erythropoietin in a normal subject.* Clin Endocrinol (Oxf), 1982. **17**(4): p. 419-22.
47. Le Hir, M., et al., *Structure-function correlations in erythropoietin formation and oxygen sensing in the kidney.* Klin Wochenschr, 1991. **69**(13): p. 567-75.
48. Miller, C.B., et al., *Decreased erythropoietin response in patients with the anemia of cancer.* N Engl J Med, 1990. **322**(24): p. 1689-92.
49. Spivak, J.L., et al., *Serum immunoreactive erythropoietin in HIV-infected patients.* JAMA, 1989. **261**(21): p. 3104-7.
50. Koury, S.T., et al., *Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration.* Blood, 1989. **74**(2): p. 645-51.
51. Abbrecht, P.H. and J.K. Littell, *Plasma erythropoietin in men and mice during acclimatization to different altitudes.* J Appl Physiol, 1972. **32**(1): p. 54-8.

52. Eckardt KU, D.J., Neumann R, Bauer C, Kurtz A. , *Decline of erythropoietin formation at continuous hypoxia is not due to feedback inhibition.* American Journal of Physiology, 1990.
53. Goldberg, M.A., C.C. Gaut, and H.F. Bunn, *Erythropoietin mRNA levels are governed by both the rate of gene transcription and posttranscriptional events.* Blood, 1991. **77**(2): p. 271-7.
54. Rondon, I.J., et al., *Hypoxia up-regulates the activity of a novel erythropoietin mRNA binding protein.* J Biol Chem, 1991. **266**(25): p. 16594-8.
55. McGary, E.C., I.J. Rondon, and B.S. Beckman, *Post-transcriptional regulation of erythropoietin mRNA stability by erythropoietin mRNA-binding protein.* J Biol Chem, 1997. **272**(13): p. 8628-34.
56. Eckardt, K.U., et al., *Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia.* J Appl Physiol (1985), 1989. **66**(4): p. 1785-8.
57. Fraser, J.K., F.K. Lin, and M.V. Berridge, *Expression and modulation of specific, high affinity binding sites for erythropoietin on the human erythroleukemic cell line K562.* Blood, 1988. **71**(1): p. 104-9.
58. Anagnostou, A., et al., *Erythropoietin Has a Mitogenic and Positive Chemotactic Effect on Endothelial-Cells.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(15): p. 5978-5982.
59. Anagnostou, A., et al., *Erythropoietin receptor mRNA expression in human endothelial cells.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3974-8.
60. Digicaylioglu, M., et al., *Localization of specific erythropoietin binding sites in defined areas of the mouse brain.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3717-20.
61. Sawyer, S.T., S.B. Krantz, and K. Sawada, *Receptors for erythropoietin in mouse and human erythroid cells and placenta.* Blood, 1989. **74**(1): p. 103-9.
62. Yamazaki, T., et al., *Effect of erythropoietin on Leydig cell is associated with the activation of Stat5 pathway.* Molecular and Cellular Endocrinology, 2004. **213**(2): p. 193-198.
63. Ammarguellat F, L.M., Kelly PA, Goffin V., *Low doses of EPO activate MAP kinases but not JAK2-STAT5 in rat vascular smooth muscle cells.* Biochem Biophys Res Commun, 2001.
64. Sinclair, A.M., et al., *Functional erythropoietin receptor is undetectable in endothelial, cardiac, neuronal, and renal cells.* Blood, 2010. **115**(21): p. 4264-4272.
65. Elliott, S., et al., *Lack of expression and function of erythropoietin receptors in the kidney.* Nephrol Dial Transplant, 2012. **27**(7): p. 2733-45.

66. Anagnostou, A.L., E.S.; Kessimian, N.; Levinson, R.; Steiner, M., *Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells.* Proc. Natl. Acad. Sci. USA 87, 5978–5982., 1990.
67. Carlini, R.G., et al., *Effect of recombinant human erythropoietin on endothelial cell apoptosis.* Kidney Int, 1999. **55**(2): p. 546-53.
68. Chong, Z.Z. and K. Maiese, *Erythropoietin involves the phosphatidylinositol 3-kinase pathway, 14-3-3 protein and FOXO3a nuclear trafficking to preserve endothelial cell integrity.* Br J Pharmacol, 2007. **150**(7): p. 839-50.
69. Beleslin-Cokic, B.B., et al., *Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells.* Blood, 2004. **104**(7): p. 2073-80.
70. Arcasoy, M.O., *Non-erythroid effects of erythropoietin.* Haematologica, 2010. **95**(11): p. 1803-5.
71. Ruschitzka, F.T., et al., *Nitric oxide prevents cardiovascular disease and determines survival in polyglobulic mice overexpressing erythropoietin.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11609-13.
72. Kanagy, N.L., et al., *Erythropoietin administration in vivo increases vascular nitric oxide synthase expression.* J Cardiovasc Pharmacol, 2003. **42**(4): p. 527-33.
73. Quaschning, T., et al., *Erythropoietin-induced excessive erythrocytosis activates the tissue endothelin system in mice.* FASEB J, 2003. **17**(2): p. 259-61.
74. Zhang, Y., et al., *Erythropoietin action in stress response, tissue maintenance and metabolism.* Int J Mol Sci, 2014. **15**(6): p. 10296-333.
75. Martinez-Estrada, O.M., et al., *Erythropoietin protects the in vitro blood-brain barrier against VEGF-induced permeability.* Eur J Neurosci, 2003. **18**(9): p. 2538-44.
76. Kertesz, N., et al., *The role of erythropoietin in regulating angiogenesis.* Dev Biol, 2004. **276**(1): p. 101-10.
77. Santhanam, A.V., et al., *In vivo stimulatory effect of erythropoietin on endothelial nitric oxide synthase in cerebral arteries.* Am J Physiol Heart Circ Physiol, 2006. **291**(2): p. H781-6.
78. Teng, R.F., et al., *Acute erythropoietin cardioprotection is mediated by endothelial response.* Basic Research in Cardiology, 2011. **106**(3): p. 343-354.
79. Cai, Z., et al., *Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury.* Circulation, 2003. **108**(1): p. 79-85.
80. Calvillo, L., et al., *Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4802-6.

81. Fiordaliso, F., et al., *A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia-reperfusion injury.* Proc Natl Acad Sci U S A, 2005. **102**(6): p. 2046-51.
82. Rafiee, P., et al., *Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways.* Basic Res Cardiol, 2005. **100**(3): p. 187-97.
83. Burger, D., et al., *Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase.* Cardiovasc Res, 2006. **72**(1): p. 51-9.
84. Bennett, C.L., et al., *Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia.* JAMA, 2008. **299**(8): p. 914-24.
85. Feldman, L., et al., *Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells.* Prostate, 2006. **66**(2): p. 135-45.
86. Kumar, S.M., et al., *Erythropoietin activates the phosphoinositide 3-kinase/Akt pathway in human melanoma cells.* Melanoma Res, 2006. **16**(4): p. 275-83.
87. Westenfelder, C. and R.L. Baranowski, *Erythropoietin stimulates proliferation of human renal carcinoma cells.* Kidney Int, 2000. **58**(2): p. 647-57.
88. Brines, M., *The therapeutic potential of erythropoiesis-stimulating agents for tissue protection: a tale of two receptors.* Blood Purif, 2010. **29**(2): p. 86-92.
89. Bernaudin, M., et al., *A potential role for erythropoietin in focal permanent cerebral ischemia in mice.* J Cereb Blood Flow Metab, 1999. **19**(6): p. 643-51.
90. Imamura, R., et al., *Carbamylated erythropoietin improves angiogenesis and protects the kidneys from ischemia-reperfusion injury.* Cell Transplant, 2008. **17**(1-2): p. 135-41.
91. Brines, M.L., et al., *Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10526-31.
92. Syed RS1, R.S., Li C, Cheetham JC, Aoki KH, Liu B, Zhan H, Osslund TD, Chirino AJ, Zhang J, Finer-Moore J, Elliott S, Sitney K, Katz BA, Matthews DJ, Wendoloski JJ, Egrie J, Stroud RM., *Efficiency of signalling through cytokine receptors depends critically on receptor orientation.* Nature, 1998.
93. Uversky, V.N. and E.M. Redwan, *Erythropoietin and co.: intrinsic structure and functional disorder.* Mol Biosyst, 2016. **13**(1): p. 56-72.
94. Syed, R.S., et al., *Efficiency of signalling through cytokine receptors depends critically on receptor orientation.* Nature, 1998. **395**(6701): p. 511-516.
95. Takeuchi, M., et al., *Role of sugar chains in the in vitro biological activity of human erythropoietin produced in recombinant Chinese hamster ovary cells.* J Biol Chem, 1990. **265**(21): p. 12127-30.

96. Yuen, C.T., et al., *Relationships between the N-glycan structures and biological activities of recombinant human erythropoietins produced using different culture conditions and purification procedures.* British Journal of Haematology, 2003. **121**(3): p. 511-526.
97. Cheetham, J.C., Smith, D.M., Aoki, K.H., Stevenson, J.L., Hoeffel, T.J., Syed, R.S., Egrie, J., Harvey, T.S., *HUMAN ERYTHROPOIETIN, NMR MINIMIZED AVERAGE STRUCTURE.* Nat Struct Biol 5 861-866 1998.
98. Erbayraktar, S., et al., *Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(11): p. 6741-6746.
99. Wen D, B.J., Tracy TE, GruningerRH, MulcahyLS, Czelusniak J, Goodman M, Bunn HF., *Erythropoietin structure-function relationships:High degree of sequence homology among mammals.* Blood 82: 1507-1516, 1993.
100. Chu, C.Y., et al., *Erythropoietins from teleosts.* Cell Mol Life Sci, 2008. **65**(22): p. 3545-52.
101. Nogawa-Kosaka, N., et al., *Structural and biological properties of erythropoietin in Xenopus laevis.* Exp Hematol, 2010. **38**(5): p. 363-72.
102. Watowich, S.S., *The erythropoietin receptor: molecular structure and hematopoietic signaling pathways.* J Investig Med, 2011. **59**(7): p. 1067-72.
103. Syed, R.S., Reid, S.W., Li, C., Cheetham, J.C., Aoki, K.H., Liu, B., Zhan, H., Osslund, T.D., Chirino, A.J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B.A., Matthews, D.J., Wendoloski, J.J., Egrie, J., Stroud, R.M., *Efficiency of signalling through cytokine receptors depends critically on receptor orientation.* Nature 395: 511-516, 1998.
104. Rose, A.S., et al., *NGL viewer: web-based molecular graphics for large complexes.* Bioinformatics, 2018. **34**(21): p. 3755-3758.
105. Watowich, S.S., et al., *Homodimerization and constitutive activation of the erythropoietin receptor.* Proc Natl Acad Sci U S A, 1992. **89**(6): p. 2140-4.
106. Philo, J.S., et al., *Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: one high-affinity and one low-affinity interaction.* Biochemistry, 1996. **35**(5): p. 1681-91.
107. Matthews, D.J., et al., *A sequential dimerization mechanism for erythropoietin receptor activation.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9471-6.
108. Zhang, Y.L., et al., *Symmetric signaling by an asymmetric 1 erythropoietin: 2 erythropoietin receptor complex.* Mol Cell, 2009. **33**(2): p. 266-74.

109. Huang LJ, C.S., Lodish HF *The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor.* Mol Cell. 8:1327–1338., 2001.
110. Parganas E, W.D., Stravopodis D, Topham DJ, Marine J-C, Teglund S, Vanin EF, Bodner S,Colamonici OR, van Deursen JM, *Jak2 is essential for signaling through a variety of cytokine receptors.* Cell. 93:385–395, 1998.
111. Jelkmann, W., *Regulation of Erythropoietin production.* J Physiolo, 2011.
112. Curt M. Horvath, Z.W., and James E. Darnell Jr. , *A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain* GENES & DEVELOPMENT 9:984-994 1995.
113. Alvarez, J.V. and D.A. Frank, *Genome-wide analysis of STAT target genes: elucidating the mechanism of STAT-mediated oncogenesis.* Cancer Biol Ther, 2004. **3**(11): p. 1045-50.
114. Newmeyer DD, B.-W.E., Kluck RM, Wolf BB, Beere HM, Green DR., *Bcl-xL does not inhibit the function of Apaf-1.* Cell Death Differ. 7:402–7. , 2000.
115. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
116. Jelkmann, W., *Physiology and pharmacology of erythropoietin.* Transfus Med Hemother, 2013. **40**(5): p. 302-9.
117. Sasaki, A., et al., *CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2.* J Biol Chem, 2000. **275**(38): p. 29338-47.
118. Supino-Rosin, L., et al., *A cytosolic domain of the erythropoietin receptor contributes to endoplasmic reticulum-associated degradation.* Eur J Biochem, 1999. **263**(2): p. 410-9.
119. Yoshimura A, L.G., Lodish HF., *Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormoneindependent activation and tumorigenicity.* Nature 348: 647-649, 1990.
120. Bondurant, M.C. and M.J. Koury, *Anemia induces accumulation of erythropoietin mRNA in the kidney and liver.* Mol Cell Biol, 1986. **6**(7): p. 2731-3.
121. Dame, C. and S.E. Juul, *The switch from fetal to adult erythropoiesis.* Clin Perinatol, 2000. **27**(3): p. 507-26.
122. Creasy, R.K., et al., *Determination of fetal, placental and neonatal blood volumes in the sheep.* Circ Res, 1970. **27**(4): p. 487-94.
123. Koury, S.T., et al., *Localization of cells producing erythropoietin in murine liver by in situ hybridization.* Blood, 1991. **77**(11): p. 2497-503.
124. Schuster, S.J., et al., *Cellular sites of extrarenal and renal erythropoietin production in anaemic rats.* Br J Haematol, 1992. **81**(2): p. 153-9.

125. Eckardt, K.U., et al., *Oxygen-dependent expression of the erythropoietin gene in rat hepatocytes in vitro.* Pflugers Arch, 1993. **423**(5-6): p. 356-64.
126. Maxwell P.H. , F.D.J.P., Osmond M.K. , Pugh C.W. , Heryet A. , Doe B.G. , Johnson M.H. , and Ratcliffe P.J. , *Expression of a Homologously Recombined Erythopoietin-SV40 T Antigen Fusion Gene in Mouse Liver: Evidence for Erythropoietin Production by Ito Cells* Blood; pp 1823-1830 1994. **84**.
127. Ramadori, G., *The stellate cell (Ito-cell, fat-storing cell, lipocyte, perisinusoidal cell) of the liver. New insights into pathophysiology of an intriguing cell.* Virchows Arch B Cell Pathol Incl Mol Pathol, 1991. **61**(3): p. 147-58.
128. Maxwell, P.H., et al., *Sites of erythropoietin production.* Kidney Int, 1997. **51**(2): p. 393-401.
129. Goldberg, M.A., et al., *The regulated expression of erythropoietin by two human hepatoma cell lines.* Proc Natl Acad Sci U S A, 1987. **84**(22): p. 7972-6.
130. Eckardt, K.U., et al., *Age-dependent expression of the erythropoietin gene in rat liver and kidneys.* J Clin Invest, 1992. **89**(3): p. 753-60.
131. Dame, C., et al., *Erythropoietin mRNA expression in human fetal and neonatal tissue.* Blood, 1998. **92**(9): p. 3218-25.
132. Boulpaep, B.a., *Medical Physiology.* Elseviers Saunders, 2005.
133. Wenger, R.H. and D. Hoogewijs, *Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells.* Am J Physiol Renal Physiol, 2010. **298**(6): p. F1287-96.
134. Hansen, A.J., et al., *Evidence against erythropoietin production by the carotid body.* Respir Physiol, 1973. **18**(1): p. 101-6.
135. Aperia, A.C., *The influence of arterial PO2 on renal tissue PO2.* Acta Physiol Scand, 1969. **75**(3): p. 353-9.
136. Leichtweiss HP, L.D., Weiss C, Baumgärtl H, Reschke W. , *The oxygen supply of the rat kidney: measurements of intrarenal pO2.* Pflugers Arch, 309, pp: 328-49, 1969.
137. Pallone, T.L., et al., *Countercurrent exchange in the renal medulla.* Am J Physiol Regul Integr Comp Physiol, 2003. **284**(5): p. R1153-75.
138. Verma, S.K. and B.A. Molitoris, *Renal endothelial injury and microvascular dysfunction in acute kidney injury.* Semin Nephrol, 2015. **35**(1): p. 96-107.
139. Zhang, W. and A. Edwards, *Oxygen transport across vasa recta in the renal medulla.* Am J Physiol Heart Circ Physiol, 2002. **283**(3): p. H1042-55.
140. Eckardt KU, K.A., Bauer C., *Regulation of erythropoietin production is related to proximal tubular function.* Am J Physiol, 1989.
141. Pagel, H., W. Jelkmann, and C. Weiss, *Erythropoietin production in the isolated perfused kidney.* Biomed Biochim Acta, 1990. **49**(2-3): p. S271-4.

142. Ratcliffe, P.J., et al., *Oxygen-dependent modulation of erythropoietin mRNA levels in isolated rat kidneys studied by RNase protection.* J Exp Med, 1990. **172**(2): p. 657-60.
143. Koury, S.T., M.C. Bondurant, and M.J. Koury, *Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization.* Blood, 1988. **71**(2): p. 524-7.
144. Kurtz, A., et al., *Site of erythropoietin formation.* Contrib Nephrol, 1989. **76**: p. 14-20; discussion 21-3.
145. Lacombe, C., et al., *Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney.* J Clin Invest, 1988. **81**(2): p. 620-3.
146. Maxwell PH, O.M., Pugh CW, Heryet A, Nicholls LG, Tan CC, and F.D. Doe BG, Johnson MH, Ratcliffe PJ., *Identification of the renal erythropoietin-producing cells using transgenic mice.* Kidney Int44: 1149-1162, , 1993.
147. Souma, T., N. Suzuki, and M. Yamamoto, *Renal erythropoietin-producing cells in health and disease.* Front Physiol, 2015. **6**: p. 167.
148. Suzuki, N., Obara,N.,andYamamoto,M., *Use of gene-manipulated mice in the study of erythropoietin gene expression.* MethodsEnzymol. 435,157–177., 2007.
149. Obara, N., Suzuki,N.,Kim,K.,Nagasawa,T.,Imagawa,S.,andYamamoto,M, *Repression via the GATA box is essential for tissue-specific erythropoietin gene expression.* Blood 111,5223–5232., 2008.
150. Asada, N., et al., *Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice.* J Clin Invest, 2011. **121**(10): p. 3981-90.
151. Yamazaki, S., et al., *A mouse model of adult-onset anaemia due to erythropoietin deficiency.* Nature Communications, 2013. **4**.
152. Souma, T., et al., *Plasticity of renal erythropoietin-producing cells governs fibrosis.* J Am Soc Nephrol, 2013. **24**(10): p. 1599-616.
153. Yamazaki, S., Souma,T.,Hirano,I.,Pan,X.,Minegishi,N.,Suzuki,N.,etal., *A mouse model of adult-onset anaemia due to erythropoietin deficiency.* Nat.Commun. 4:1950., 2013.
154. Kobayashi, H., et al., *Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin.* J Clin Invest, 2016. **126**(5): p. 1926-38.
155. Juul, S.E., et al., *Erythropoietin and erythropoietin receptor in the developing human central nervous system.* Pediatr Res, 1998. **43**(1): p. 40-9.
156. Marti, H.H., et al., *Erythropoietin gene expression in human, monkey and murine brain.* Eur J Neurosci, 1996. **8**(4): p. 666-76.
157. Marti, H.H., *Erythropoietin and the hypoxic brain.* J Exp Biol, 2004. **207**(Pt 18): p. 3233-42.

158. Marti, H.H., et al., *Detection of erythropoietin in human liquor: intrinsic erythropoietin production in the brain.* Kidney Int, 1997. **51**(2): p. 416-8.
159. Juul, S.E., Harcum, J., Li, Y. and Christensen, R. D., *Erythropoietin is present in the cerebrospinal fluid of neonates.* J. Pediatr. 130, 428-430., 1997.
160. Bernaudin, M., Bellail, A., Marti, H. H., Yvon, A., Vivien, D., Duchatelle,I., MacKenzie, E. T. and Petit, E., *Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain.* Glia 30, 271-278., 2000.
161. Yamaji, R., et al., *Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA.* Eur J Biochem, 1996. **239**(2): p. 494-500.
162. Nagai, A., et al., *Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture.* J Neuropathol Exp Neurol, 2001. **60**(4): p. 386-92.
163. Brines, M., et al., *Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(41): p. 14907-14912.
164. Zhang, F., et al., *Enhanced Delivery of Erythropoietin Across the Blood-Brain Barrier for Neuroprotection against Ischemic Neuronal Injury.* Transl Stroke Res, 2010. **1**(2): p. 113-21.
165. Chikuma, M., et al., *Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus.* Am J Physiol Endocrinol Metab, 2000. **279**(6): p. E1242-8.
166. Bernaudin, M., et al., *Neurons and astrocytes express EPO mRNA: Oxygen-sensing mechanisms that involve the redox-state of the brain.* Glia, 2000. **30**(3): p. 271-278.
167. Lewczuk, P., Hasselblatt, M., Kamrowski-Kruck, H., Heyer, A., Unzicker, and S. C., A. L. and Ehrenreich, H., *Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin.* Neuroreport 11,3485-3488., 2000.
168. Masuda, S., et al., *A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes.* J Biol Chem, 1994. **269**(30): p. 19488-93.
169. Masuda, S., et al., *Functional erythropoietin receptor of the cells with neural characteristics. Comparison with receptor properties of erythroid cells.* J Biol Chem, 1993. **268**(15): p. 11208-16.
170. Dame, C., S.E. Juul, and R.D. Christensen, *The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential.* Biol Neonate, 2001. **79**(3-4): p. 228-35.
171. Yu, X.B., et al., *Erythropoietin receptor signalling is required for normal brain development.* Development, 2002. **129**(2): p. 505-516.

172. Noguchi, C.T., et al., *Role of erythropoietin in the brain.* Crit Rev Oncol Hematol, 2007. **64**(2): p. 159-71.
173. Sakanaka, M., Wen, T. C., Matsuda, S., Masuda, S., Morishita, E., Nagao, M. and Sasaki, R., *In vivo evidence that erythropoietin protects neurons from ischemic damage.* Proc. Natl. Acad. Sci. USA 95, 4635-4640, 1998.
174. Shingo, T., et al., *Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells.* J Neurosci, 2001. **21**(24): p. 9733-43.
175. Assandri, R., et al., *Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line.* J Physiol, 1999. **516 (Pt 2)**: p. 343-52.
176. Koshimura, K., et al., *Effects of erythropoietin on neuronal activity.* J Neurochem, 1999. **72**(6): p. 2565-72.
177. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
178. Ribatti, D., et al., *Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo.* Blood, 1999. **93**(8): p. 2627-36.
179. Sugawa, M., et al., *Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation.* Neurosci Res, 2002. **44**(4): p. 391-403.
180. Marti, H.H. and W. Risau, *Angiogenesis in ischemic disease.* Thromb Haemost, 1999. **82 Suppl 1**: p. 44-52.
181. Brines, M. and A. Cerami, *Emerging biological roles for erythropoietin in the nervous system.* Nat Rev Neurosci, 2005. **6**(6): p. 484-94.
182. Anagnostou, A., Lee, E. S., Kessimian, N., Levinson, R. & Steiner, M, *Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells.* Proc.Natl Acad. Sci. USA 87, 5978–5982 1990.
183. Tojo, A., et al., *Identification of erythropoietin receptors on fetal liver erythroid cells.* Biochem Biophys Res Commun, 1987. **148**(1): p. 443-8.
184. Campana, W.M., R. Misasi, and J.S. O'Brien, *Identification of a neurotrophic sequence in erythropoietin.* Int J Mol Med, 1998. **1**(1): p. 235-41.
185. Jubinsky, P.T., et al., *The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor.* Blood, 1997. **90**(5): p. 1867-73.
186. Scott CL, R.L., Papaevangeliou B, Mansfield R, Nicola NA, Begley CG., *Reassessment of interactions between hematopoietic receptors using common beta-chain and interleukin-3-specific receptor betachain-null cells: no evidence of functional interactions with receptors for erythropoietin, granulocyte colonystimulating factor, or stem cell factor.* Blood ;96(4):1588-90., 2000.

187. Juhaszova M, Z.D., Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ., *Glycogen synthase kinase-3β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore.* J. Clin.Invest. 113, 1535–1549 2004.

188. Serhat Erbayraktar, G.G., Alessandra Sfacteria, Qiao-wen Xie, Thomas Coleman, Mads Kreilgaard, Lars Torup, Thomas Sager, Zubeyde Erbayraktar, Necati Gokmen, Osman Yilmaz, Pietro Ghezzi, Pia Villa, Maddalena Fratelli, Simona Casagrande, Marcel Leist, Lone Helboe, Jens Gerwein, Søren Christensen, Marie Aavang Geist, Lars Østergaard Pedersen, Carla Cerami-Hand, Jean-Paul Wuerth, Anthony Cerami, and Michael Brines, *Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo.* Proc. Natl Acad. Sci. USA 100, 6741–6746 2003.

189. Leist M, G.P., Grasso G, Bianchi R, Villa P, Fratelli M, Savino C, Bianchi M, Nielsen J, Gerwien J, Kallunki P, Larsen AK, Helboe L, Christensen S, Pedersen LO, Nielsen M, Torup L, Sager T, Sfacteria A, Erbayraktar S, Erbayraktar Z, Gokmen N, Yilmaz O, Cerami-Hand C, Xie QW, Coleman T, Cerami A, Brines M., *Derivatives of erythropoietin that are tissue protective but not erythropoietic.* Science 305, 239–242, 2004.

190. Taylor, C.T. and J.C. McElwain, *Ancient atmospheres and the evolution of oxygen sensing via the hypoxia-inducible factor in metazoans.* Physiology (Bethesda), 2010. **25**(5): p. 272-9.

191. Beerling, D.J. and R.A. Berner, *Feedbacks and the coevolution of plants and atmospheric CO2.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1302-5.

192. Schirrmeister, B.E., et al., *Evolution of multicellularity coincided with increased diversification of cyanobacteria and the Great Oxidation Event.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1791-6.

193. Dole, M., *The natural history of oxygen.* J Gen Physiol, 1965. **49**(1): p. Suppl:5-27.

194. Martin, W., Mentel, M. , *The Origin of Mitochondria. .* Nature Education 3(9):58 2010.

195. Alberts B., e.a., *Molecular biology of the cell, 4th Edition. New York, Garland Science* 2002.

196. Conway Morris, S., *Darwin's dilemma: the realities of the Cambrian 'explosion'.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1470): p. 1069-83.

197. Carreau A, E.H.-R.B., Matejuk A, Grillon C, Kieda C., *Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia.* J Cell Mol Med.;15(6):1239-53., 2011.

198. Wenger, R.H., et al., *Frequently asked questions in hypoxia research.* Hypoxia (Auckl), 2015. **3**: p. 35-43.

199. Cassavaugh, J. and K.M. Lounsbury, *Hypoxia-mediated biological control.* J Cell Biochem, 2011. **112**(3): p. 735-44.
200. Michiels, C., *Physiological and pathological responses to hypoxia.* Am J Pathol, 2004. **164**(6): p. 1875-82.
201. Bigham, A.W., *Genetics of human origin and evolution: high-altitude adaptations.* Curr Opin Genet Dev, 2016. **41**: p. 8-13.
202. Hooper T, M.A., *Cardiovascular physiology at high altitude.* J R Army Med Corps., 2011.
203. Honig, A., *Peripheral arterial chemoreceptors and reflex control of sodium and water homeostasis.* Am J Physiol, 1989. **257**(6 Pt 2): p. R1282-302.
204. Kapoor, R., A.S. Narula, and A.C. Anand, *Treatment of Acute Mountain Sickness and High Altitude Pulmonary Oedema.* Med J Armed Forces India, 2004. **60**(4): p. 384-7.
205. Azad, P., et al., *High-altitude adaptation in humans: from genomics to integrative physiology.* J Mol Med (Berl), 2017. **95**(12): p. 1269-1282.
206. Beall, C.M., *Adaptation to high altitude: phenotypes and genotypes.* Annual Review of Anthropology, 2014.
207. Winslow, R.M., et al., *Different hematologic responses to hypoxia in Sherpas and Quechua Indians.* J Appl Physiol (1985), 1989. **66**(4): p. 1561-9.
208. Chiodi, H., *Respiratory adaptations to chronic high altitude hypoxia.* J Appl Physiol, 1957. **10**(1): p. 81-7.
209. Zhuang, J., et al., *Hypoxic ventilatory responsiveness in Tibetan compared with Han residents of 3,658 m.* J Appl Physiol (1985), 1993. **74**(1): p. 303-11.
210. Beall CM, S.K., Blangero J, Williams-Blangero S, Almasy LA, Decker MJ, Worthman CM, Goldstein MC, Vargas E, Villena M, Soria R, Alarcon AM, Gonzales C., *Ventilation and Hypoxic Ventilatory Response of Tibetanand Aymara High Altitude Natives.* Am J Phys Anthropol.114(1):42-53., 1997.
211. Bigham, A.W. and F.S. Lee, *Human high-altitude adaptation: forward genetics meets the HIF pathway.* Genes Dev, 2014. **28**(20): p. 2189-204.
212. Arias-Stella, J. and M. Saldana, *The muscular pulmonary arteries in people native to high altitude.* Med Thorac, 1962. **19**: p. 484-93.
213. Groves, B.M., et al., *Minimal hypoxic pulmonary hypertension in normal Tibetans at 3,658 m.* J Appl Physiol (1985), 1993. **74**(1): p. 312-8.
214. Moore LG, Z.S., Zhuang J, Sun S, Droma T., *Oxygen transport in tibetan women during pregnancy at 3,658 m.* Am J Phys Anthropol.114(1):42-53., 2001.
215. Lorenzo, F.R., et al., *A genetic mechanism for Tibetan high-altitude adaptation.* Nat Genet, 2014. **46**(9): p. 951-6.

216. Huerta-Sanchez, E., et al., *Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA.* Nature, 2014. **512**(7513): p. 194-7.
217. Zhou, D., et al., *Whole-genome sequencing uncovers the genetic basis of chronic mountain sickness in Andean highlanders.* Am J Hum Genet, 2013. **93**(3): p. 452-62.
218. Scheinfeldt, L.B., et al., *Genetic adaptation to high altitude in the Ethiopian highlands.* Genome Biol, 2012. **13**(1): p. R1.
219. Wenger, R.H., *Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression.* FASEB J, 2002. **16**(10): p. 1151-62.
220. Hampton-Smith, R.J. and D.J. Peet, *From polyps to people: a highly familiar response to hypoxia.* Ann N Y Acad Sci, 2009. **1177**: p. 19-29.
221. Fukuda, R., et al., *HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells.* Cell, 2007. **129**(1): p. 111-22.
222. Semenza, G.L. and G.L. Wang, *A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.* Mol Cell Biol, 1992. **12**(12): p. 5447-54.
223. Wang, G.L. and G.L. Semenza, *Purification and characterization of hypoxia-inducible factor 1.* J Biol Chem, 1995. **270**(3): p. 1230-7.
224. Lisy, K. and D.J. Peet, *Turn me on: regulating HIF transcriptional activity.* Cell Death Differ, 2008. **15**(4): p. 642-9.
225. Makino, Y., et al., *Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.* Nature, 2001. **414**(6863): p. 550-4.
226. Epstein, A.C., et al., *C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.* Cell, 2001. **107**(1): p. 43-54.
227. Ohh, M., et al., *Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein.* Nat Cell Biol, 2000. **2**(7): p. 423-7.
228. Arany, Z., et al., *An essential role for p300/CBP in the cellular response to hypoxia.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 12969-73.
229. Lando, D., et al., *Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch.* Science, 2002. **295**(5556): p. 858-61.
230. Lu, H., et al., *Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1.* J Biol Chem, 2005. **280**(51): p. 41928-39.
231. Zhao, S., et al., *Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1alpha.* Science, 2009. **324**(5924): p. 261-5.
232. Fong, G.H. and K. Takeda, *Role and regulation of prolyl hydroxylase domain proteins.* Cell Death Differ, 2008. **15**(4): p. 635-41.

233. Stiehl, D.P., et al., *Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system.* J Biol Chem, 2006. **281**(33): p. 23482-91.
234. Paliege, A., et al., *Hypoxia-inducible factor-2alpha-expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization.* Kidney Int, 2010. **77**(4): p. 312-8.
235. Rosenberger, C., et al., *Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys.* J Am Soc Nephrol, 2002. **13**(7): p. 1721-32.
236. Wiesener, M.S., et al., *Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs.* FASEB J, 2003. **17**(2): p. 271-3.
237. Minamishima, Y.A., et al., *A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo.* Mol Cell Biol, 2009. **29**(21): p. 5729-41.
238. Iyer, N.V., et al., *Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha.* Genes Dev, 1998. **12**(2): p. 149-62.
239. Tian H, H.R., Matsumoto AM, Russell DW, McKnight SL., *The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development.* Genes Dev., 1998.
240. Compernolle, V., et al., *Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice.* Nat Med, 2002. **8**(7): p. 702-10.
241. Scortegagna, M., et al., *Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1(-/-) mice.* Nature Genetics, 2003. **35**(4): p. 331-340.
242. Scortegagna M, D.K., Zhang Q, Oktay Y, Bennett MJ, Bennett M, Shelton JM, Richardson JA, Moe O, Garcia JA., *HIF-2α regulates murine hematopoietic development in an erythropoietin-dependent manner. .* Blood, 2005.
243. Bishop, T., et al., *Abnormal sympathoadrenal development and systemic hypotension in PHD3-/- mice.* Mol Cell Biol, 2008. **28**(10): p. 3386-400.
244. Amandine Thomas, E.B., Judith Aron-Wisnewsky, Gerard C. van der Zon, Patrick Levy, Karine Clement, Jean-Louis Pepin, Diane Godin-Ribuot & Bruno Guigas, *Hypoxia-inducible factor prolyl hydroxylase 1 (PHD1) deficiency promotes hepatic steatosis and liver-specific insulin resistance in mice.* Scientific Reports, 2016.
245. Minamishima, Y.A., et al., *Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure.* Blood, 2008. **111**(6): p. 3236-44.
246. Takeda, K., et al., *Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins.* Blood, 2008. **111**(6): p. 3229-35.

247. Takeda, K., A. Cowan, and G.H. Fong, *Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system.* Circulation, 2007. **116**(7): p. 774-81.
248. Maher ER, N.H., Richard S., *von Hippel-Lindau disease: a clinical and scientific review.* Eur J Hum Genet. 2011 2011.
249. Hickey MM, L.J., Bezman NA, Rathmell WK, Simon MC., *von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis.* J Clin Invest, 2007.
250. Wiesener, M.S., et al., *Paraneoplastic erythrocytosis associated with an inactivating point mutation of the von Hippel-Lindau gene in a renal cell carcinoma.* Blood, 2002. **99**(10): p. 3562-5.
251. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE.* Sci STKE, 2005. **2005**(306): p. re12.
252. Firth, J.D., B.L. Ebert, and P.J. Ratcliffe, *Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements.* J Biol Chem, 1995. **270**(36): p. 21021-7.
253. Wenger, R.H., et al., *Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site.* Eur J Biochem, 1998. **253**(3): p. 771-7.
254. Smythies, J.A., et al., *Inherent DNA-binding specificities of the HIF-1alpha and HIF-2alpha transcription factors in chromatin.* EMBO Rep, 2019. **20**(1).
255. Beru, N., et al., *Expression of the erythropoietin gene.* Mol Cell Biol, 1986. **6**(7): p. 2571-5.
256. Semenza, G.L., et al., *Human erythropoietin gene expression in transgenic mice: multiple transcription initiation sites and cis-acting regulatory elements.* Mol Cell Biol, 1990. **10**(3): p. 930-8.
257. Ebert, B.L. and H.F. Bunn, *Regulation of the erythropoietin gene.* Blood, 1999. **94**(6): p. 1864-77.
258. Shoemaker, C.B. and L.D. Mitsock, *Murine erythropoietin gene: cloning, expression, and human gene homology.* Mol Cell Biol, 1986. **6**(3): p. 849-58.
259. Galson, D.L., et al., *Comparison of the human and mouse erythropoietin genes shows extensive homology in the flanking regions.* Blood, 1993. **82**(11): p. 3321-6.
260. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5680-4.

261. Kochling, J., P.T. Curtin, and A. Madan, *Regulation of human erythropoietin gene induction by upstream flanking sequences in transgenic mice.* Br J Haematol, 1998. **103**(4): p. 960-8.
262. Haidar, M.A., et al., *Differential expression of lacZ in the liver and kidney of transgenic mice carrying chimeric lacZ-erythropoietin gene constructs with or without its 1.2 kb 3'-flanking sequence.* Nucleic Acids Res, 1996. **24**(18): p. 3621-8.
263. Suzuki, N., et al., *Specific contribution of the erythropoietin gene 3′ enhancer to hepatic erythropoiesis after late embryonic stages.* Molecular and Cellular Biology, 2011. **31**(18): p. 3896-3905.
264. Suzuki, N., et al., *Erythropoietin production in neuroepithelial and neural crest cells during primitive erythropoiesis.* Nature Communications, 2013. **4**: p. 2902.
265. Madan, A. and P.T. Curtin, *A 24-base-pair sequence 3' to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 3928-32.
266. Blanchard, K.L., et al., *Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements.* Mol Cell Biol, 1992. **12**(12): p. 5373-85.
267. Gupta, M. and E. Goldwasser, *The role of the near upstream sequence in hypoxia-induced expression of the erythropoietin gene.* Nucleic Acids Res, 1996. **24**(23): p. 4768-74.
268. Fandrey, J., *Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression.* Am J Physiol Regul Integr Comp Physiol, 2004. **286**(6): p. R977-88.
269. Imagawa, S., et al., *Regulatory elements of the erythropoietin gene.* Blood, 1991. **77**(2): p. 278-85.
270. Shimizu, R. and M. Yamamoto, *Gene expression regulation and domain function of hematopoietic GATA factors.* Semin Cell Dev Biol, 2005. **16**(1): p. 129-36.
271. Imagawa, S., et al., *GATA suppresses erythropoietin gene expression through GATA site in mouse erythropoietin gene promoter.* Int J Hematol, 2002. **75**(4): p. 376-81.
272. Imagawa, S., M. Yamamoto, and Y. Miura, *Negative regulation of the erythropoietin gene expression by the GATA transcription factors.* Blood, 1997. **89**(4): p. 1430-9.
273. Dame, C., et al., *Hepatic erythropoietin gene regulation by GATA-4.* J Biol Chem, 2004. **279**(4): p. 2955-61.
274. Kaneko, H., et al., *Induction of erythropoietin gene expression in epithelial cells by chemicals identified in GATA inhibitor screenings.* Genes Cells, 2017. **22**(11): p. 939-952.
275. Gupta, M., P.T. Mungai, and E. Goldwasser, *A new transacting factor that modulates hypoxia-induced expression of the erythropoietin gene.* Blood, 2000. **96**(2): p. 491-7.

276. Lee, S.B. and D.A. Haber, *Wilms tumor and the WT1 gene.* Exp Cell Res, 2001. **264**(1): p. 74-99.
277. Dame, C., et al., *Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene.* Blood, 2006. **107**(11): p. 4282-90.
278. Pugh, C.W., et al., *Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(23): p. 10553-7.
279. Storti, F., et al., *A novel distal upstream hypoxia response element regulating oxygen-dependent erythropoietin gene expression.* Haematologica, 2014. **99**(4): p. e45-8.
280. Galson, D.L., et al., *The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1.* Mol Cell Biol, 1995. **15**(4): p. 2135-44.
281. Rankin, E.B., et al., *Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo.* J Clin Invest, 2007. **117**(4): p. 1068-77.
282. Suzuki, N., et al., *Specific contribution of the erythropoietin gene 3' enhancer to hepatic erythropoiesis after late embryonic stages.* Mol Cell Biol, 2011. **31**(18): p. 3896-905.
283. Stolze, I., et al., *Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells.* Blood, 2002. **100**(7): p. 2623-8.
284. Yin, H. and K.L. Blanchard, *DNA methylation represses the expression of the human erythropoietin gene by two different mechanisms.* Blood, 2000. **95**(1): p. 111-9.
285. Steinmann, K., A.M. Richter, and R.H. Dammann, *Epigenetic silencing of erythropoietin in human cancers.* Genes Cancer, 2011. **2**(1): p. 65-73.
286. Chang, Y.T., et al., *DNA methyltransferase inhibition restores erythropoietin production in fibrotic murine kidneys.* J Clin Invest, 2016. **126**(2): p. 721-31.
287. McMullin, M.F., et al., *Guidelines for the diagnosis, investigation and management of polycythaemia/erythrocytosis.* Br J Haematol, 2005. **130**(2): p. 174-95.
288. Bento, C., et al., *Genetic basis of congenital erythrocytosis: mutation update and online databases.* Hum Mutat, 2014. **35**(1): p. 15-26.
289. Keohane, C., M.F. McMullin, and C. Harrison, *The diagnosis and management of erythrocytosis.* BMJ, 2013. **347**: p. f6667.
290. Hoyer JD, A.S., Beutler E, Kubik K, West C, Fairbanks VF., *Erythrocytosis due to bisphosphoglycerate mutase deficiency with concurrent glucose-6-phosphate dehydrogenase (G-6-PD) deficiency.* Am J Hematol., 2004.
291. Ang, S.O., et al., *Endemic polycythemia in Russia: mutation in the VHL gene.* Blood Cells Mol Dis, 2002. **28**(1): p. 57-62.

292. Petousi, N., et al., *Erythrocytosis associated with a novel missense mutation in the BPGM gene.* Haematologica, 2014. **99**(10): p. e201-4.
293. Hand, H., *Blood and the classification of anaemia.* Nurs Stand, 2001. **15**(39): p. 45-53; quiz 54-5.
294. El Nahas, M., *The global challenge of chronic kidney disease.* Kidney Int, 2005. **68**(6): p. 2918-29.
295. Caro J, B.S., Miller O, Murray T, Erslev AJ., *Erythropoietin levels in uremic nephric and anephric patients.* J Lab Clin Med., 2004.
296. Chandra, M., G.K. Clemons, and M.I. McVicar, *Relation of serum erythropoietin levels to renal excretory function: evidence for lowered set point for erythropoietin production in chronic renal failure.* J Pediatr, 1988. **113**(6): p. 1015-21.
297. Brookhart, M.A., et al., *The effect of altitude on dosing and response to erythropoietin in ESRD.* J Am Soc Nephrol, 2008. **19**(7): p. 1389-95.
298. Frede, S., et al., *Erythropoietin gene expression is suppressed after lipopolysaccharide or interleukin-1 beta injections in rats.* Am J Physiol, 1997. **273**(3 Pt 2): p. R1067-71.
299. Maxwell, P.H. and K.U. Eckardt, *HIF prolyl hydroxylase inhibitors for the treatment of renal anaemia and beyond.* Nat Rev Nephrol, 2016. **12**(3): p. 157-68.
300. Bernhardt, W.M., et al., *Donor treatment with a PHD-inhibitor activating HIFs prevents graft injury and prolongs survival in an allogenic kidney transplant model.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21276-81.
301. Paliege A, R.C., Bondke A, Sciesielski L, Shina A, Heyman SN, Flippin LA, Arend M, Klaus SJ, Bachmann S., *Hypoxia-inducible factor-2alpha-expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization.* Kidney Int 77: 312–318 2010.
302. Dhillon, S., *Roxadustat: First Global Approval.* Drugs, 2019. **79**(5): p. 563-572.
303. Wang, F., et al., *RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues.* J Mol Diagn, 2012. **14**(1): p. 22-9.

2. Aims of the thesis

The availability of hepatoma and neuroblastoma cell lines helped to gain new insights into Epo regulation, whereas a reliable cell culture model derived from renal Epo-producing (REP) cells is currently lacking. While hepatic Epo expression is known to be regulated by the 3' HRE, the regulatory elements modulating Epo transcriptional activation in brain and kidney remain unknown. A kidney inducible region (KIE) located between -14 and -6 kb from TSS of *EPO* has been shown to be responsible for hypoxic-inducible renal Epo. Within this KIE, we previously identified a novel 5' HRE having similar features to the 3' HRE and could show that both HREs direct exogenous reporter gene expression. However, the functional relevance of this HRE for endogenous Epo induction remained unknown.

In this PhD work, I aim for the investigation of the transcriptional regulation of Epo in hypoxia. In particular, I am interested in:

I. Dissecting the contribution of the endogenous distal 5' and 3' HREs as well as the promoter to endogenous *EPO* gene expression in hepatoma and neuroblastoma cell lines.
II. Generating a novel transgenic mouse model to conditionally tag for active *Epo* locus and isolate fresh renal Epo producing cells.
III. Detecting Epo mRNA at single cell level by highly specific and sensitive *in situ* hybridization method (RNA scope) to analyse cell-to-cell variability of the "hypoxic transcript pattern", defined as the cellular distribution of HIF induced single nascent and mature mRNA molecules in hepatoma, neuroblastoma and FAIK cell line.

3. Distal and proximal hypoxia response elements cooperate to regulate organ-specific erythropoietin gene expression

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ABSTRACT

While it is well-established that distal hypoxia response elements (HREs) regulate hypoxia-inducible factor (HIF) target genes such as erythropoietin (Epo), an interplay between multiple distal and proximal (promoter) HREs has not been described so far. Hepatic Epo expression is regulated by a HRE located downstream of the *EPO* gene, but this 3' HRE is dispensable for renal *EPO* gene expression. We previously identified a 5' HRE and could show that both HREs direct exogenous reporter gene expression. Here, we show that whereas in hepatic cells the 3' but not the 5' HRE is required, in neuronal cells both the 5' and 3' HREs contribute to endogenous Epo induction. Moreover, two novel putative HREs were identified in the *EPO* promoter. In hepatoma cells HIF interacted mainly with the distal 3' HRE, but in neuronal cells HIF most strongly bound the promoter, to a lesser extent the 3' HRE, and not at all the 5' HRE. Interestingly, mutation of either of the two distal HREs abrogated HIF binding to the 3' and promoter HREs. These results suggest that a canonical functional HRE can recruit multiple, not necessarily HIF, transcription factors to mediate HIF binding to different distant HREs in an organ-specific manner.

Article Summary

Hypoxia-inducible EPO gene expression is regulated by a 3' HRE in the liver but this element is not required for EPO induction in other organs and the DNA elements required for EPO regulation remained elusive. Using CRISPR-Cas9 mediated gene editing we could demonstrate that a novel 5' HRE - despite not binding HIF - functionally cooperates with the 3' HRE and newly discovered promoter HREs to direct inducible and organ-specific EPO gene expression.

INTRODUCTION

Erythropoietin (Epo) is indispensable for the maintenance of a normal blood oxygen concentration and Epo synthesis is induced under anemic and hypoxic conditions (1,2). In the adult, Epo production by the kidney and liver account for approx. 90% and 10%, respectively, of total circulating Epo, but ectopic Epo expression was also found in the brain, uterus and testis, among others (3-6). Renal Epo is synthesized by peritubular pericytes with fibroblastic and neuronal features, located in the juxtamedullary cortex (7-9). These cells respond to a decrease in tissue oxygen partial pressure by hypoxia-inducible factor (HIF)-2 stabilization and HIF-2-dependent transcriptional induction of *EPO* gene expression (10). HIFs bind to a hypoxia response element (HRE) containing the essential core consensus sequence 5'-RCGTG-3' which, however, is not sufficient to confer hypoxia-inducible gene expression (11). Distinct regulatory DNA elements enhance *EPO* promoter activity in liver and kidney. Transgenic mouse models showed that in the liver 0.7 kb of the immediate 3'-

flanking region is required, whereas in the kidney the essential regulatory element resides between -14 and -6 kb in the distal 5'-region (12-15). The 3' HRE is well established and has been shown to be necessary and sufficient for liver-specific *EPO* gene expression after embryonic day 14.5 (16,17). However, the DNA element responsible for kidney-specific *EPO* gene expression is far less well characterized and nothing is known about the endogenous HREs in Epo-producing cells of other tissues.

We recently discovered a strongly conserved distal 5' HRE and suggested that it might contribute to oxygen-regulated *EPO* expression (18). This 5' HRE resides within a DNaseI hypersensitive site -9.2 kb upstream of the *EPO* transcriptional start site, contains both the 5'-ACGTG-3' core HIF DNA binding site as well as the ancillary 5'-CACA-3' element (11), and confers hypoxia-inducible exogenous reporter gene expression in Epo expressing and non-expressing cell lines (18). However, the organ-specific relevance of the endogenous 5' and 3' HREs as well as their functional interaction with the *EPO* promoter remained unknown. Considering the neuronal features that we and others reported for the renal Epo-producing cells (8,9), we hence analyzed the relative contribution of the 5' and 3' HREs in Kelly cells, a human neuroblastoma cell line that has previously been shown to regulate the *EPO* gene in an oxygen and HIF dependent manner (19). Endogenous HRE function was investigated by gene editing and HIF-DNA interaction studies. Intriguingly, novel promoter HREs were identified and profound differences in the functional cooperation between the 5', 3' and promoter HREs in Kelly cells were found when compared with the well-established human Hep3B hepatoma cell model (20).

MATERIALS AND METHODS

Cell culture

Human Hep3B and HepG2 hepatocellular carcinoma (American Type Culture Collection, LGC Standards, Wesel, Germany) and Kelly neuroblastoma (kindly provided by J. Fandrey, Essen, Germany) cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium, respectively (Sigma Aldrich, Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA USA), 50 IU/ml penicillin and 50 µg/ml streptomycin (Sigma Aldrich, Saint Louis, MO, USA). Cells were exposed to hypoxic conditions using a workstation as described previously (21).

Gene editing

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) gene editing was performed as described (22) and is described in details in the Supplementary Methods.

RNA and protein analyses

RNA was extracted and quantified by reverse-transcription (RT) real-time quantitative (q) PCR as described previously (21,25). In brief, RT was performed with 2 µg total RNA and AffinityScript reverse transcriptase (Agilent), and the cDNA quantified using SYBR Green qPCR reagent kit (Kapa Biosystems, London, UK) in a MX3000P light cycler (Agilent). Transcripts levels were calculated through comparison with calibrated standard curves and normalized to human ribosomal protein L28 mRNA. Primers used for RT-qPCR are listed in Supplementary Table 1. Epo protein was detected by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Immunoblotting was performed as described previously (21) using the following primary antibodies: mouse monoclonal anti-HIF-1α (#610959; BD Transduction Laboratories, San Jose, CA, USA), rabbit monoclonal anti-HIF-2α (#PAB12124; Abnova, Taipei, Taiwan), mouse monoclonal anti-HIF-α (D28F3; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-β-actin (A5441; Sigma Aldrich). Secondary antibodies were HRP-conjugated goat polyclonal anti-rabbit or anti-mouse IgG (#31460 and #31430, respectively; Thermo Fisher Scientific).

Luciferase reporter gene assays

Reporter gene constructs have been described previously (18). Canonical 5'-RCGTG-3' promoter HREs were replaced by 5'-RAAAG-3' using site-directed mutagenesis. Following transfection with lipofectamine, cells were incubated under normoxic or hypoxic (0.2% O_2) conditions for 24 hours. Reporter gene assays were performed as described before (21) using a luciferase assay kit (Promega, Madison, Wisconsin, USA). Luciferase activity was normalized to the protein content as determined by the Bradford assay (26).

HIF chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (21,27,28), using the following rabbit polyclonal antibodies: anti-HIF-1α (PM14), anti-HIF-2α (PM9), anti-HIF-β (NB100-110; Novus Biologicals, Littleton, CO, USA), normal rabbit serum (X0902; Dako, Glostrup, Denmark). Co-precipitated DNA was quantified by real-time qPCR using the primers listed in Supplementary Table 1.

Statistical analysis

All data are shown as mean + SEM. Unpaired two-tailed Student's t-tests were applied. Differences between two values at the $p<0.05$ level were considered to be statistically significant.

RESULTS

Mutation of the *EPO* 5' and 3' HREs by gene editing

CRISPR-Cas9 technology was used for the specific destruction of the endogenous *EPO* HREs to dissect the relative contribution of the -9.2 kb 5' HRE and the +3.0 kb 3' HRE to hypoxia-inducible *EPO* gene expression in neuronal and hepatic cell lines (Figure 1A). As shown in Figure 1B, sgRNAs were designed to target the HIF-binding core sequence 5'-ACGTG-3' (11). The restriction enzyme TaiI was used to assess the presence of the 5'-ACGT-3' sequence (24). Successful HRE destruction confers TaiI resistance to the PCR products as exemplified for Kelly cells in Figures 1C and D. Monoclonal cell lines were obtained by limiting dilution cloning of initial partially gene edited polyclonal cell pools. The HRE regions were again amplified by PCR, re-tested for complete TaiI resistance, and cloned into plasmid vectors. Multiple independent plasmids were sequenced to ensure biallelic HRE inactivation. Alternatively, amplicons were directly deep sequenced. Clone verification and sequence information is provided in Supplementary Figure 1. Note that in some cases (Kelly-3'B2, Hep3B-5'H11) more than two mutant alleles were detected which may be due to either polyclonal cell lines, polyploidy, gene amplification or genetic drift. Other clones maintained wild-type HRE sequences (Kelly-5'C4, Kelly-3'C4, Hep3B-5'A5, HepG2-5'H8, HepG2-3'D6; confirmed by sequencing of PCR products) and were included as additional controls in subsequent experiments.

The 5' HRE contributes to hypoxic *EPO* induction in neuronal but not hepatic cells

The neuroblastoma cell line Kelly has been reported to induce endogenous Epo mRNA levels, at 24 hours, by over 71-fold under hypoxic conditions (3% O_2) and over 238-fold under anoxic conditions (19). Kelly cells hence recapitulate the well-known hypoxia-inducible *EPO* gene expression in the brain (4). As shown in Figure 2, Epo mRNA and secreted protein were induced by 1603-fold and 22-fold, respectively, following exposure of wild-type Kelly cells to hypoxia (0.2% O_2) for 24 hours. However, due to the very low basal Epo transcript levels, the fold induction factor is subject to variation and must be interpreted with caution. Indeed, the wild-type HRE subclones Kelly-5'C4 and Kelly-3'C4 showed reduced Epo mRNA induction, while the hypoxic Epo mRNA levels were not significantly different from the maternal Kelly cells (Figure 2A). These results were confirmed on the protein level where the normoxic levels also varied greatly but the hypoxic levels were indistinguishable from the maternal cell line (Figure 2B). Therefore, only hypoxic Epo levels were considered for subsequent analyses.

Interestingly, all mutant 5' and 3' HRE Kelly clones showed significantly reduced hypoxic Epo expression, on the mRNA (Figure 2A) as well as on the protein (Figure 2B) level. This is in striking contrast to mutant Hep3B (Figure 2C, D) and HepG2 (Figure 2E)

clones, where only 3' but not 5' HRE mutations strongly reduced hypoxic Epo mRNA and protein levels. Note that while Kelly and Hep3B cells were exposed to hypoxia for 24 hours, HepG2 cells showed maximal Epo mRNA induction already after 8 hours, with approx. 10-fold lower mRNA levels, which was not sufficient for detectable Epo protein accumulation in the supernatant. To ensure that no general off-target effects caused these results, the mRNA levels of the HIF-1 and HIF-2 target genes *CAIX* and *LOXL2* (Kelly) or *PAI1* (Hep3B and HepG2), respectively (29), were measured in the same samples. As shown in Supplementary Figure 2, *EPO* HRE mutations did not significantly reduce the expression of these genes under hypoxic conditions. Moreover, also hypoxic HIF-1α and HIF-2α protein stabilization was not altered by the *EPO* HRE mutations (Supplementary Figure 3). These results suggest that both the 5' and 3' HREs contribute to hypoxia-inducible *EPO* gene expression in neuronal cells whereas in hepatic cells only the 3' HRE is required.

The 5' and 3' *EPO* HREs cooperatively enhance hypoxic reporter gene expression in neuronal cells

We had previously shown that the *EPO* 5' HRE confers hypoxia-inducible expression to a heterologous SV40 promoter-driven reporter gene in both Hep3B and Kelly cells (18). These results stand in apparent contrast to the 5' HRE mutation data presented above and imply differences between exogenous bacterial reporter gene plasmids and endogenous chromatin regulation. In fact, similar reporter gene results have been obtained even in non-Epo-expressing HeLa and HK-2 cells (18) as well as in Hek293 cells (data not shown). We hence expanded these experiments by transiently transfecting Kelly cells with minimal (138 bp) *EPO* promoter-driven reporter genes, enhanced by various DNA fragments containing the 5' and/or 3' HREs. In contrast to our previous results obtained with Hep3B cells (18), *EPO* promoter-driven luciferase activity in Kelly cells was significantly further elevated if the 100 bp fragment containing the 5' HRE was combined with a 126 bp fragment containing the 3' HRE (Figure 3A). While a longer 3 kb DNA fragment containing the 5' HRE reduced both hypoxic and normoxic reporter gene expression driven by the *EPO* promoter/3' HRE, it actually led to even higher hypoxic induction factors. These data imply a cooperation between the *EPO* 5' and 3' HREs specifically in neuronal cells, and suggest that additional distal and proximal 5' flanking elements contribute to tissue-specific and conditional *EPO* regulation.

A novel *EPO* promoter HRE contributes to hypoxic reporter gene expression in neuronal cells

Interestingly, a slight but significant hypoxic induction of the minimal *EPO* promoter could be seen in Kelly cells (Figure 3A) which we previously did not observe in Hep3B cells (18).

Inspection of the 147 bp fragment containing the 138 bp *EPO* promoter revealed a tandem dimeric repeat with two previously not reported putative HREs (Figure 3B). These putative HREs are highly conserved and locate close to Wt1 and GATA binding sites (Supplementary Figure 4). In combination with the 5' and 3' HRE enhancers, luciferase expression driven by this promoter fragment was induced 152-fold by hypoxia in this experimental series. Mutation of either promoter HRE 1 (pHRE1) or pHRE2 significantly reduced hypoxic luciferase expression levels, whereas the effect on the hypoxic induction factors was less conclusive (Figure 3C). Double pHRE1 and pHRE2 mutation did not further decrease reporter gene expression, suggesting that this tandem dimeric sequence in the *EPO* promoter acts as a single HRE.

HIF interacts with the *EPO* promoter and 3' HRE but not 5' HRE in neuronal cells

To directly analyze the interaction of HIF with the various HREs of the endogenous *EPO* locus, chromatin immunoprecipitation followed by real-time PCR quantification (ChIP-qPCR) experiments were performed. Because of the contribution of the promoter to the hypoxic activation of reporter gene expression observed above, we also included the proximal 5' region in these ChIP-qPCR experiments (Figure 4A). Unexpectedly, Kelly cells did not show any significant binding of the HIF subunits HIF-1α, HIF-2α or HIF-β to the 5' HRE, whereas a significant hypoxic increase in HIF-2α /HIF-β binding to the 3' HRE could be detected (Figure 4B). Surprisingly, however, the strongest HIF interaction in hypoxic Kelly cells was observed with the *EPO* promoter region, again with a preference for HIF-2α /HIF-β. In contrast, in hypoxic Hep3B cells the strongest HIF-2α/HIF-β interaction was found at the 3' HRE and only a weak interaction with the promoter region could be observed (Figure 4C). To control for ChIP efficiency, the presence of DNA fragments containing the HRE of the HIF-1/2 target genes *NDRG1* and *PAI1* (21,27) was quantified by qPCR in the same samples. HIF binding to the established *NDRG1* and *PAI1* promoter HREs was comparable to the *Epo* HREs, and anti-HIF-β antibodies precipitated roughly additive amounts of chromatin seen with the HIF-1α and HIF-2α antibodies (Figures 4B, C).

Functional distal 3' and 5' HREs are required for remote control of HIF binding to the *EPO* promoter

Because ChIP resolution is insufficient to detect the precise protein-DNA interaction site, we next wanted to confirm that HIF actually bound the core sequence of the investigated HREs. Therefore, Hep3B 3' and 5' mutant HRE subclones were exposed to hypoxia and used for ChIP with anti-HIF-β antibodies to precipitate both HIF-1 and HIF-2. As expected, HIF bound the wild-type 3' HRE but did not interact anymore with the mutant 3' HRE (Figure 4D). Of note, the 3' HRE mutation apparently also decreased the weak HIF interaction with the *EPO*

promoter region. Consistent with its non-functionality in Hep3B cells, mutation of the 5' HRE did not influence HIF binding to any of the tested HREs (Figure 4D). HIF-β binding to the *PAI1* control HRE (Figure 4D) as well as HIF-β protein levels (Figure 4E) were not altered in these Hep3B subclones, confirming that HIF-β expression is not generally affected by the 3' and 5' HRE mutations and that HIF-β ChIP reliably detects HIF-DNA interactions.

To further investigate the unexpected inhibitory effect of the distal 3' HRE mutation on the HIF interaction with the proximal *EPO* promoter region in Hep3B cells, these experiments were repeated with Kelly 3' and 5' mutant HRE subclones. Intriguingly, the 3' HRE mutation (clone B2) not only completely abrogated the interaction between HIF and the 3' HRE, but also strongly decreased the interaction with the promoter region (Figure 5A). Moreover, the 5' HRE mutation (clone C3) similarly impaired HIF interaction with the 3' HRE and the promoter region (Figure 5A), while comparable HIF binding to the *NDRG1* control HRE (Figure 5B) and HIF-β protein levels (Figure 5C) could be detected in these mutant Kelly subclones.

Large but not small deletions of the 5' HRE abrogate Epo induction in 3'/5' HRE double-mutant neuronal cells

Because there was residual HIF binding to the *EPO* promoter in the single 3' and 5' HRE mutant Kelly subclones, we wondered whether 3'/5' HRE double mutations would lead to a further decrease of the HIF interaction with the *EPO* promoter. The introduction of a secondary mutation was rather inefficient and worked only in the B2 3' HRE mutant Kelly subclone. Deep sequencing of the PCR products derived from the mutant 5' HRE revealed 3 subclones with large (31 bp and 18 bp; varying ratios) and 3 subclones with small (1, 2 or 3 bp; biallelic) deletions (Supplementary Figure 5A). Despite some reduction in CAIX mRNA and HIF-1α protein in the subclones containing the larger deletions, the double-mutant subclones did not show major general differences in mRNA levels of the HIF target genes CAIX and LOXL2, or in hypoxic HIF-1α and HIF-2α protein levels (Supplementary Figures 5B-E).

In hypoxic Kelly cells, only the larger but not the smaller 5' HRE deletions further decreased HIF binding to the *EPO* promoter in the 3'/5' HRE double-mutant subclones (Figure 5A). The small core 5' HRE mutations partly reversed the inhibitory effect of the 3' HRE mutation on HIF promoter interaction. HIF-β binding to the *NDRG1* control HRE (Figure 5B) as well as HIF-β protein levels (Figure 5C) remained at similar levels, confirming that HIF-β expression is not generally affected in these Kelly subclones, and that HIF-β ChIP reliably detects HIF-DNA interactions. Consistent with the ChIP data, the additional larger or smaller 5' HRE deletions further decreased or partly reversed, respectively, Epo mRNA (Figure 5D) and protein (Figure 5E) levels in hypoxic 3' HRE mutant Kelly cells.

DISCUSSION

The data presented reveal a complex and organ-specific interplay between various HREs at the *EPO* locus. The previously described mechanisms of *EPO* regulation in hepatic cells were mostly confirmed by our experiments, including a strong functional dependence on the endogenous 3' HRE with a robust HIF interaction, and a complete lack of requirement for the endogenous 5' HRE despite a strong hypoxic enhancer function in exogenous reporter gene experiments. In neuronal cells, the endogenous 5' HRE seems to be as important as the 3' HRE for hypoxic induction of *EPO* gene expression and the 5' and 3' HREs cooperate in hypoxic enhancement of exogenous reporter gene expression, but the 5' HRE does not directly interact with HIF. In contrast to hepatic cells, where the *EPO* promoter is only slightly bound by HIF and does not confer hypoxic reporter gene induction (18), in neuronal cells it is the *EPO* promoter that most strongly interacts with HIF, consistent with a weak hypoxic induction of reporter genes. *EPO* minimal promoter fragments spanning at least -91 bp upstream of the transcriptional start site have previously been shown to be hypoxia responsive in reporter gene experiments also in Hep3B cells (30,31).

It is currently unclear why we repeatedly found significant hypoxic promoter induction only in Kelly but not Hep3B cells. Inspection of the minimal *EPO* promoter region revealed two potential HREs characterized by the presence of evolutionary conserved HIF core 5'-GCGTG-3' binding motifs. Mutation of either of these two HREs in Kelly cells reduced both basal and hypoxia-inducible promoter activity enhanced by the 5' and 3' HREs. Tissue-specific transcriptional co-activators and/or co-repressors may be involved in the difference in hypoxic promoter activity and HIF binding between hepatic and neuronal cells. Indeed, a cooperative interaction with additional transcription factors binding in close proximity to HIF is typical for HREs and has also been found in other HIF target genes (21). Specifically, while the ancillary 5'-CACA-3' element (11) is missing, its supportive function may be replaced by the neighbouring DNA binding sites for GATA factors and Wilms tumor suppressor (Wt1), which are well-known to regulate the *EPO* promoter (32-35). Alternatively, it is possible that binding of HIF to the promoter and/or transcriptional activation may be blocked by binding of a currently unknown tissue-specific factor in Hep3B cells. Of note, we have previously shown that ATF/CREB family members are able to directly interact with the HRE core motif (36), and it may be that such an interaction also occurs at the promoter HREs.

How could it be explained that a conserved HRE consensus core sequence, including the ancillary element, is functionally relevant for hypoxic induction of gene expression but not directly bound by HIF, as it appears to be the case for the *EPO* 5' HRE? We have previously shown for *PAG1*, another HIF-2 target gene, that a single distal -82 kb 5' HRE resides in an isolated DNA region, bound by many additional transcription factors, and forms multiple chromatin loops both locally and over a long distance with the promoter region (21). While in

this case HIF-2α did interact with the HRE, neither hypoxia nor the presence of HIF was needed for the long-range chromatin interaction with the promoter region. Only the presence of the core 5'-ACGTG-3' HIF binding DNA sequence was required to maintain this interaction, suggesting that pre-formed chromatin loops enable oxygen-regulated conditional gene regulation. This model has subsequently been confirmed for many other HIF target genes by genome-wide approaches (37,38). Therefore, the *EPO* 5' HRE might well be functionally required for hypoxia-inducible gene expression by maintaining a constitutive chromatin architecture that supports promoter activity in a cell type-specific manner. The finding that only additional large but not small 5' HRE deletions fully abrogated endogenous Epo induction and HIF:promoter interaction in 3' HRE mutant Kelly cells suggests that, like in the case of the *PAG1* gene, additional transcription factors bind close to the consensus HRE sequence and are involved in chromatin looping and *trans*-activation of the *EPO* promoter. We have currently no explanation why a small 5' HRE deletion alone inhibited HIF:promoter interaction, but in combination with a 3' HRE mutation partially rescued the inhibitory effect of the 3' HRE mutation. Nonetheless, it is without precedent that the extended 5' HRE strongly *cis*-enhanced HIF binding to the promoter and 3' HREs.

The differences between Epo-producing hepatic and neuronal cells raise the question of how *EPO* is regulated in renal Epo-producing (REP) cells, the main source of circulating Epo. The overlap of neuronal markers with genetically tagged mouse REP cells (8,9) suggests that Kelly cells may represent a better model than hepatoma cells to recapitulate human oxygen-regulated *EPO* gene expression in the kidney, at least concerning the enigmatic 5' regulatory regions. Indeed, deletion of the endogenous 3' HRE in the mouse suppressed only hepatic but not renal *Epo* expression, suggesting the presence of one or more additonal HREs in REP cells (17). Consistently, deletion of a -17.4 to -3.6 *Epo* 5' region in transgenic mouse models abrogated *Epo* gene expression specifically in REP cells whereas it was dispensable for *Epo* expression in the brain (31). Mutation of the mouse -8.3 kb 5' HRE (corresponds to the human -9.2 kb 5' HRE) within this *Epo* 5' region, did not abrogate transgenic *Epo* gene expression in the kidney (brain was not analyzed) and a minimal 0.3 kb fragment containing this 5' HRE was not sufficient to drive transgenic GFP expression in the mouse kidney (31). Our results on the cooperation between 5', 3' and promoter HREs in neuronal and hepatic cell lines may help to explain these findings in transgenic mice: while the deletion of the 3' HRE in the liver is sufficient to abrogate hypoxic Epo expression, only the combined deletion of the extended 5' and the minimal 3' (and maybe the promoter) HREs may affect Epo expression in the kidney.

Altogether, these results demonstrate that several HREs are involved in oxygen-regulated *EPO* gene expression. While in the liver the 3' HRE is both necessary and sufficient, in the kidney the 3' HRE is dispensable and a 5' HRE acts in concert with

additional long-range enhancer elements and probably local chromatin structure. Although transgenic mouse models are still lacking, our results suggest that in the brain, both of these distal HREs functionally cooperate with HIF binding to promoter HREs. Our results further illustrate that not all HREs act in a canonical way by directly binding HIF, and that HRE-promoter cooperations in oxygen-regulated gene expression need to be analyzed in a cell type-specific context and cannot be generalized based on the results obtained with a single cell line. Similar results have recently been reported for the *MALAT1* locus, expressing a lncRNA which is strongly hypoxia-inducible (39), where HIF-dependent and independent long-range interactions contribute to hypoxia-inducibility in a cell-type specific manner (40). Finally, our findings are also relevant for disease-associated polymorphisms that either create or delete potential HREs (28,41,42) and may hence interfere with long-range chromatin interactions.

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REFERENCES

1. Wenger, R.H. and Hoogewijs, D. (2010) Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. Am. J. Physiol. Renal. Physiol., 298, F1287-1296.

2. Wenger, R.H. and Kurtz, A. (2011) Erythropoietin. Compr. Physiol., 1, 1759-1794.

3. Tan, C.C., Eckardt, K.U. and Ratcliffe, P.J. (1991) Organ distribution of erythropoietin messenger RNA in normal and uremic rats. Kidney Int., 40, 69-76.

4. Marti, H.H., Wenger, R.H., Rivas, L.A., Straumann, U., Digicaylioglu, M., Henn, V., Yonekawa, Y., Bauer, C. and Gassmann, M. (1996) Erythropoietin gene expression in human, monkey and murine brain. Eur. J. Neurosci., 8, 666-676.

5. Yasuda, Y., Masuda, S., Chikuma, M., Inoue, K., Nagao, M. and Sasaki, R. (1998) Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. J. Biol. Chem., 273, 25381-25387.

6. Magnanti, M., Gandini, O., Giuliani, L., Gazzaniga, P., Marti, H.H., Gradilone, A., Frati, L., Agliano, A.M. and Gassmann, M. (2001) Erythropoietin expression in primary rat Sertoli and peritubular myoid cells. Blood, 98, 2872-2874.

7. Maxwell, P.H., Osmond, M.K., Pugh, C.W., Heryet, A., Nicholls, L.G., Tan, C.C., Doe, B.G., Ferguson, D.J., Johnson, M.H. and Ratcliffe, P.J. (1993) Identification of the renal erythropoietin-producing cells using transgenic mice. Kidney Int., 44, 1149-1162.

8. Obara, N., Suzuki, N., Kim, K., Nagasawa, T., Imagawa, S. and Yamamoto, M. (2008) Repression via the GATA box is essential for tissue-specific erythropoietin gene expression. Blood, 111, 5223-5232.

9. Imeri, F., Nolan, K.A., Bapst, A.M., Santambrogio, S., Abreu-Rodríguez, I., Spielmann, P., Pfundstein, S., Libertini, S., Crowther, L., Orlando, I.M.C. et al. (2019) Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation, and a telocyte phenotype. Kidney Int., 95, 375-387.

10. Kapitsinou, P.P., Liu, Q., Unger, T.L., Rha, J., Davidoff, O., Keith, B., Epstein, J.A., Moores, S.L., Erickson-Miller, C.L. and Haase, V.H. (2010) Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. Blood, 116, 3039-3048.

11. Wenger, R.H., Stiehl, D.P. and Camenisch, G. (2005) Integration of oxygen signaling at the consensus HRE. Sci. STKE, 2005, re12.

12. Semenza, G.L., Dureza, R.C., Traystman, M.D., Gearhart, J.D. and Antonarakis, S.E. (1990) Human erythropoietin gene expression in transgenic mice: multiple transcription initiation sites and cis-acting regulatory elements. Mol. Cell. Biol., 10, 930-938.

13. Semenza, G.L., Nejfelt, M.K., Chi, S.M. and Antonarakis, S.E. (1991) Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc. Natl. Acad. Sci. U.S.A., 88, 5680-5684.

14. Madan, A., Lin, C., Hatch, S.L.I. and Curtin, P.T. (1995) Regulated basal, inducible, and tissue-specific human erythropoietin gene expression in transgenic mice requires multiple cis DNA sequences. Blood, 85, 2735-2741.

15. Köchling, J., Curtin, P.T. and Madan, A. (1998) Regulation of human erythropoietin gene induction by upstream flanking sequences in transgenic mice. Br. J. Haematol., 103, 960-968.

16. Semenza, G.L. and Wang, G.L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol., 12, 5447-5454.

17. Suzuki, N., Obara, N., Pan, X., Watanabe, M., Jishage, K., Minegishi, N. and Yamamoto, M. (2011) Specific contribution of the erythropoietin gene 3' enhancer to hepatic erythropoiesis after late embryonic stages. Mol. Cell. Biol., 31, 3896-3905.

18. Storti, F., Santambrogio, S., Crowther, L., Otto, T., Abreu-Rodríguez, I., Kaufmann, M., Hu, C.J., Dame, C., Fandrey, J., Wenger, R.H. et al. (2014) A novel distal upstream hypoxia response element regulating oxygen-dependent erythropoietin gene expression. Haematologica, 99, e45-e48.

19. Stolze, I., Berchner-Pfannschmidt, U., Freitag, P., Wotzlaw, C., Rössler, J., Frede, S., Acker, H. and Fandrey, J. (2002) Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells. Blood, 100, 2623-2628.

20. Goldberg, M.A., Glass, G.A., Cunningham, J.M. and Bunn, H.F. (1987) The regulated expression of erythropoietin by two human hepatoma cell lines. Proc. Natl. Acad. Sci. U.S.A., 84, 7972-7976.

21. Schörg, A., Santambrogio, S., Platt, J.L., Schödel, J., Lindenmeyer, M.T., Cohen, C.D., Schrödter, K., Mole, D.R., Wenger, R.H. and Hoogewijs, D. (2015) Destruction of a distal hypoxia response element abolishes trans-activation of the PAG1 gene mediated by HIF-independent chromatin looping. Nucl. Acids Res., 43, 5810-5823.

22. Harms, D.W., Quadros, R.M., Seruggia, D., Ohtsuka, M., Takahashi, G., Montoliu, L. and Gurumurthy, C.B. (2014) Mouse genome editing using the CRISPR/Cas system. Current protocols in human genetics, 83, 15.17.11-27.

23. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nature protocols, 8, 2281-2308.

24. Wenger, R.H., Kvietikova, I., Rolfs, A., Camenisch, G. and Gassmann, M. (1998) Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site. Eur. J. Biochem., 253, 771-777.

25. Wollenick, K., Hu, J., Kristiansen, G., Schraml, P., Rehrauer, H., Berchner-Pfannschmidt, U., Fandrey, J., Wenger, R.H. and Stiehl, D.P. (2012) Synthetic transactivation screening reveals ETV4 as broad coactivator of hypoxia-inducible factor signaling. Nucl. Acids Res., 40, 1928-1943.

26. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

27. Schödel, J., Oikonomopoulos, S., Ragoussis, J., Pugh, C.W., Ratcliffe, P.J. and Mole, D.R. (2011) High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood, 117, e207-217.

28. Schödel, J., Bardella, C., Sciesielski, L.K., Brown, J.M., Pugh, C.W., Buckle, V., Tomlinson, I.P., Ratcliffe, P.J. and Mole, D.R. (2012) Common genetic variants at the 11q13.3 renal cancer susceptibility locus influence binding of HIF to an enhancer of cyclin D1 expression. Nat. Genet., 44, 420-425, S421-422.

29. Stiehl, D.P., Bordoli, M.R., Abreu-Rodríguez, I., Wollenick, K., Schraml, P., Gradin, K., Poellinger, L., Kristiansen, G. and Wenger, R.H. (2012) Non-canonical HIF-2 function drives

autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop. Oncogene, 31, 2283-2297.

30. Blanchard, K.L., Acquaviva, A.M., Galson, D.L. and Bunn, H.F. (1992) Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol. Cell. Biol., 12, 5373-5385.

31. Hirano, I., Suzuki, N., Yamazaki, S., Sekine, H., Minegishi, N., Shimizu, R. and Yamamoto, M. (2017) Renal anemia model mouse established by transgenic rescue with an erythropoietin gene lacking kidney-specific regulatory elements. Mol. Cell. Biol., 37, e00451-00416.

32. Imagawa, S., Yamamoto, M. and Miura, Y. (1997) Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood, 89, 1430-1439.

33. Imagawa, S., Suzuki, N., Ohmine, K., Obara, N., Mukai, H.Y., Ozawa, K., Yamamoto, M. and Nagasawa, T. (2002) GATA suppresses erythropoietin gene expression through GATA site in mouse erythropoietin gene promoter. Int. J. Hematol., 75, 376-381.

34. Dame, C., Sola, M.C., Lim, K.C., Leach, K.M., Fandrey, J., Ma, Y., Knopfle, G., Engel, J.D. and Bungert, J. (2004) Hepatic erythropoietin gene regulation by GATA-4. J. Biol. Chem., 279, 2955-2961.

35. Dame, C., Kirschner, K.M., Bartz, K.V., Wallach, T., Hussels, C.S. and Scholz, H. (2006) Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. Blood, 107, 4282-4290.

36. Kvietikova, I., Wenger, R.H., Marti, H.H. and Gassmann, M. (1995) The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. Nucl. Acids Res., 23, 4542-4550.

37. Platt, J.L., Salama, R., Smythies, J., Choudhry, H., Davies, J.O., Hughes, J.R., Ratcliffe, P.J. and Mole, D.R. (2016) Capture-C reveals preformed chromatin interactions between HIF-binding sites and distant promoters. EMBO Rep., 17, 1410-1421.

38. Niskanen, H., Tuszynska, I., Zaborowski, R., Heinäniemi, M., Yla-Herttuala, S., Wilczynski, B. and Kaikkonen, M.U. (2017) Endothelial cell differentiation is encompassed by changes in long range interactions between inactive chromatin regions. Nucl. Acids Res.

39. Lelli, A., Nolan, K.A., Santambrogio, S., Goncalves, A.F., Schönenberger, M.J., Guinot, A., Frew, I.J., Marti, H.H., Hoogewijs, D. and Wenger, R.H. (2015) Induction of long noncoding RNA MALAT1 in hypoxic mice. Hypoxia, 3, 45-52.

40. Stone, J.K., Kim, J.H., Vukadin, L., Richard, A., Giannini, H.K., Lim, S.S., Tan, M. and Ahn, E.E. (2019) Hypoxia induces cancer cell-specific chromatin interactions and increases MALAT1 expression in breast cancer cells. J. Biol. Chem., doi: 10.1074/jbc.RA1118.006889.

41. Grampp, S., Platt, J.L., Lauer, V., Salama, R., Kranz, F., Neumann, V.K., Wach, S., Stohr, C., Hartmann, A., Eckardt, K.U. et al. (2016) Genetic variation at the 8q24.21 renal cancer susceptibility locus affects HIF binding to a MYC enhancer. Nat. Commun., 7, 13183.

42. Grampp, S., Schmid, V., Salama, R., Lauer, V., Kranz, F., Platt, J.L., Smythies, J., Choudhry, H., Goppelt-Struebe, M., Ratcliffe, P.J. et al. (2017) Multiple renal cancer susceptibility polymorphisms modulate the HIF pathway. PLoS Genet, 13, e1006872.

FIGURE LEGENDS

Figure 1. *EPO* 5' and 3' HRE gene editing. (**A**) Scheme depicting the human *EPO* locus (HRE, hypoxia response element; HSS, hypersensitivity site; TSS, transcriptional start site; KIE, kidney inducible element; NRE, negative regulatory element; LIE, liver inducible element; NRLE, negative regulatory liver element). (**B**) Single-guided (sg) RNA sequences designed to target the *EPO* 5' and 3' HREs by CRISPR-Cas9 (bold, consensus HIF binding site; arrows, PCR primers used to amplify the HRE regions; fwd, forward; rev, reverse; wt, wild-type; mt, mutant). Mutation of the consensus HRE confers resistance to TaiI restriction digestion as shown by agarose gel analysis of digested or undigested PCR products using as template genomic DNA isolated from Kelly neuroblastoma cells following 5' (**C**) or 3' (**D**) HRE gene editing (M, marker; PL, polyclonal pool of cells; B4 and C3, 5' HRE mutant clones; T1 and F3, 3' HRE mutant clones; ctr, non-template control).

Figure 2. Hypoxia-inducible *EPO* gene expression in 5' or 3' HRE mutant neuroblastoma and hepatoma cells. *EPO* expression under normoxic or hypoxic conditions (0.2% O_2; 24 h for Kelly and Hep3B, 8 h for HepG2) was measured in Kelly (**A, B**), Hep3B (**C, D**) and HepG2 (**E**) cells by RT-qPCR on the mRNA level (**A, C, E**) and by supernatant ELISA on the protein level (**B, D**). Epo mRNA levels were normalized to ribosomal protein L28 mRNA levels. Numbers above the columns indicate hypoxic induction factors. Data are shown as mean + SEM of 3 independent experiments. Student's t-tests were used to statistically evaluate the difference to hypoxic wild-type (WT) cells (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.d., not detectable; Pool, polyclonal pool of cells; +/+, subclones containing two wild-type alleles; -/-, subclones with biallelic HRE mutation).

Figure 3. *EPO* promoter-driven and HRE-enhanced luciferase reporter gene expression. (**A, C**) Kelly cells were transiently transfected with the indicated reporter gene constructs and exposed for 24 hours to normoxic or hypoxic (0.2% O_2) conditions. Luciferase activities were normalized to the protein content and shown as mean values + SEM of 6 independent experiments. Numbers on the right of the bars indicate hypoxic induction factors. Student's t-tests were used to statistically assess the hypoxic induction of the *EPO* promoter (EpoProm, wild-type; mtpHRE, mutant) and the effects of combining the 5' with the 3' HRE (**, $p<0.01$; ***, $p<0.001$). (**B**) Sequence of the 147 bp human *EPO* promoter fragment (starting 138 bp upstream of the transcription start site; TSS), indicating a conserved tandem dimeric repeat (underlined), each containing a putative promoter HRE (pHRE1 and pHRE2).

Figure 4. HIF binding to the *EPO* locus in 5' or 3' HRE mutant neuroblastoma and hepatoma cells. (**A**) Scheme depicting the regions of the *EPO* locus analyzed for HIF interaction by chromatin immunoprecipitation (ChIP), including the 5' and 3' HREs, the minimal promoter and a negative (neg.) control region devoid of any HIF binding. Kelly (**B**) and Hep3B (**C**) cells were exposed for 24 hours to normoxic or hypoxic (0.2% O_2) conditions, followed by ChIP using antibodies derived against HIF-1α, HIF-2α or HIF-β, or a negative control serum. The promoter HRE of the established HIF target genes *NDRG1* and *PAI1* served as control for ChIP efficiency. The amount of co-precipitated DNA was determined by qPCR and displayed relative to input. (**D**) These experiments were repeated with hypoxic wild-type (WT), 5' or 3' HRE mutant Hep3B cells. Mean values + SEM of 3 (Kelly) or 4 (Hep3B) independent experiments are shown. Student's t-tests were performed to statistically evaluate the difference between the hypoxic ChIP samples and hypoxic serum controls (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$). (**E**) Immunoblot confirmation of similar HIF-β protein levels in the hypoxic Hep3B subclones used above. β-Actin was used as loading and blotting control.

Figure 5. HIF binding to the *EPO* locus and Epo regulation in 3'/5' HRE double-mutant neuroblastoma cells. (**A, B**) ChIP of hypoxic WT or mutant (as indicated) Kelly cells using antibodies derived against HIF-β or negative control serum. The amount of co-precipitated DNA was determined by qPCR covering the indicated regions of the *EPO* locus (**A**) or the HRE of the *NDRG1* control locus (**B**) and displayed relative to input. Data are shown as mean + SEM of 4 independent experiments. Student's t-tests were performed to statistically evaluate the difference to hypoxic WT (*, $p<0.05$) or 3' B2 ($^{\#}$, $p<0.05$) or 5' C3 cells (§, $p<0.05$; §§, $p<0.01$). (**C**) Immunoblot confirmation of similar HIF-β protein levels in the hypoxic Kelly subclones used above. β-Actin was used as loading and blotting control. (**D, E**) Epo production in wild-type (WT), single 3' B2 or double 3' B2 5' HRE mutant Kelly cells exposed for 24 hours to normoxia or hypoxia (0.2% O_2) was measured by RT-qPCR on the mRNA level (**D**) or by ELISA on the protein level (**E**). Data are shown as mean + SEM of n=3-6 (**D**) or n=5-10 (**E**) independent experiments. Student's t-tests were used to statistically evaluate the difference to hypoxic WT (**, $p<0.01$; ***, $p<0.001$) or 3' B2 cells ($^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$; ns, not significant; n.d., not detectable).

Figure 1

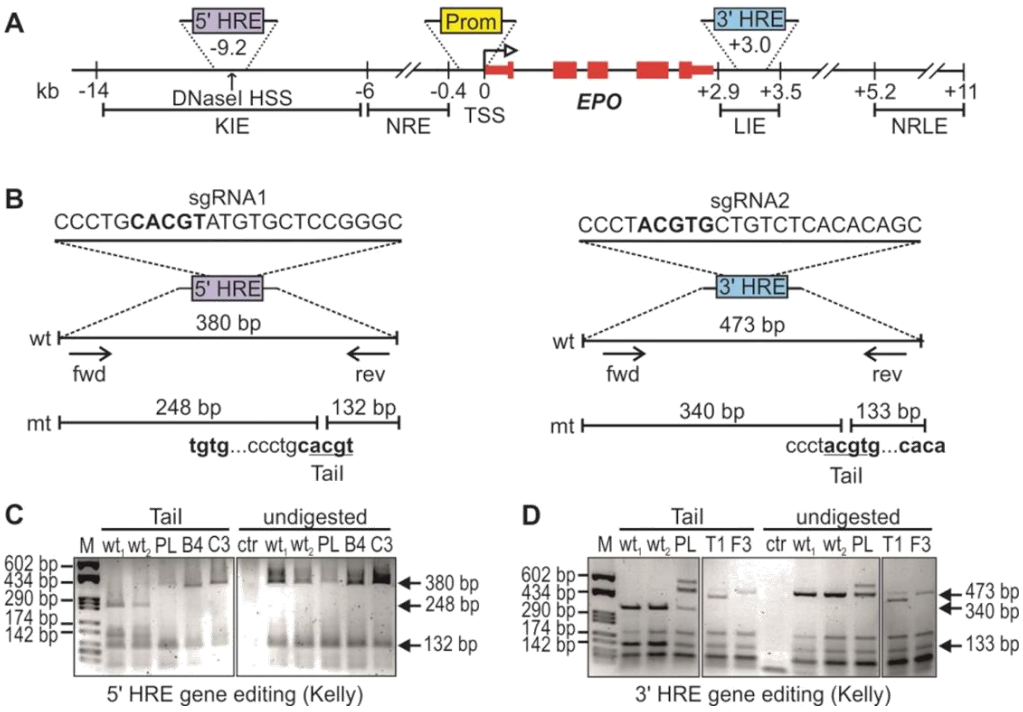

A
5' HRE
-9.2
Prom
3' HRE
+3.0
kb
-14
DNaseI HSS
-6
-0.4
0
EPO
+2.9
+3.5
+5.2
+11
KIE
NRE
TSS
LIE
NRLE
B
sgRNA1
CCCTGCACGTATGTGCTCCGGGC
5' HRE
wt
380 bp
fwd
rev
mt
248 bp
132 bp
tgtg...ccctgcacgt
Tail
sgRNA2
CCCTACGTGCTGTCTCACACAGC
3' HRE
wt
473 bp
fwd
rev
mt
340 bp
133 bp
ccctacgtg...caca
Tail
C
Tail
undigested
M wt1 wt2 PL B4 C3 ctr wt1 wt2 PL B4 C3
602 bp
434 bp
290 bp
174 bp
142 bp
380 bp
248 bp
132 bp
5' HRE gene editing (Kelly)
D
Tail
undigested
M wt1 wt2 PL T1 F3 ctr wt1 wt2 PL T1 F3
602 bp
434 bp
290 bp
174 bp
142 bp
473 bp
340 bp
133 bp
3' HRE gene editing (Kelly)

Figure 2

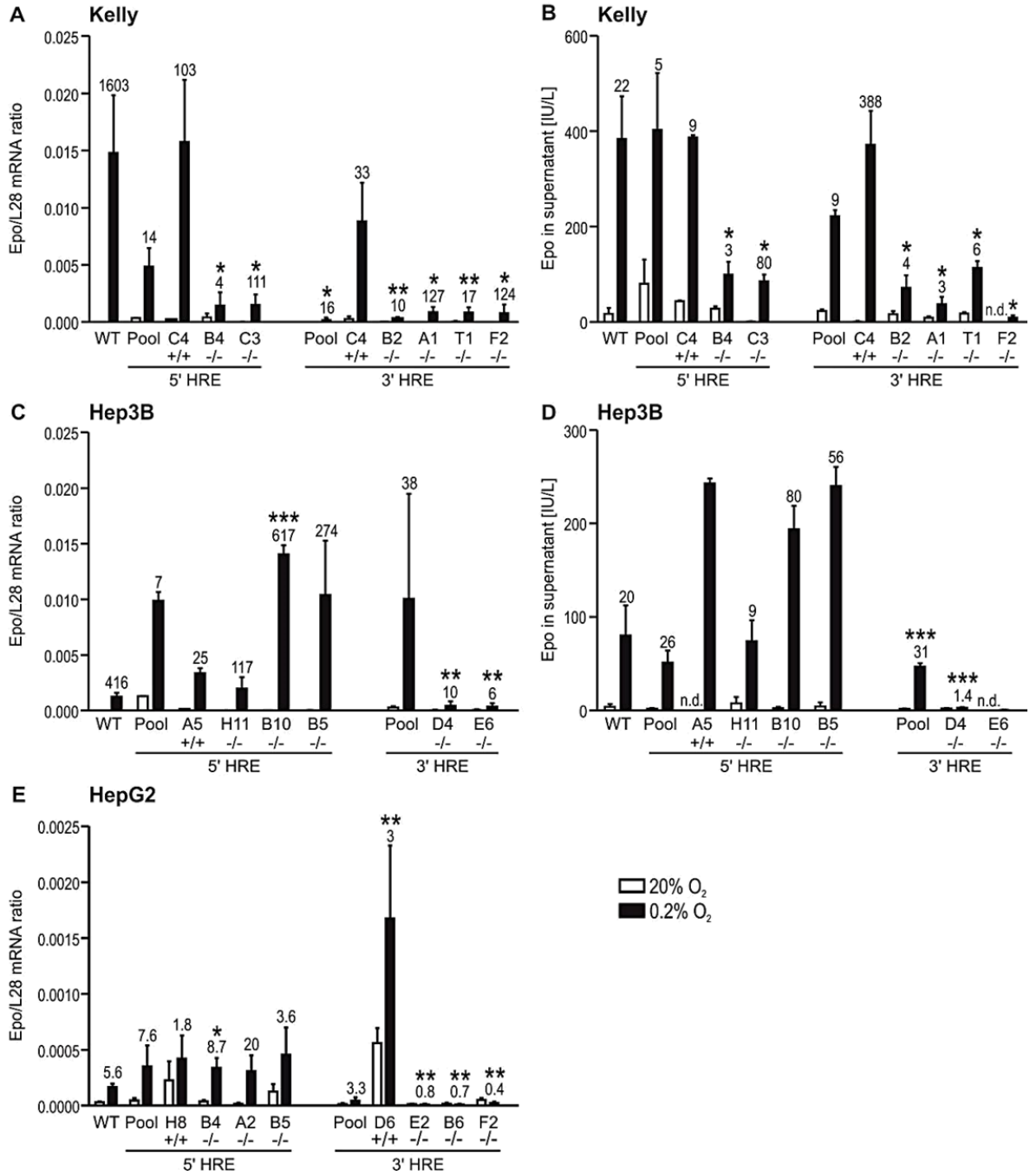

A
Kelly
Epo/L28 mRNA ratio
WT Pool C4 +/+ B4 -/- C3 -/- Pool C4 +/+ B2 -/- A1 -/- T1 -/- F2 -/-
5' HRE
3' HRE
B
Kelly
Epo in supernatant [IU/L]
C
Hep3B
WT Pool A5 +/+ H11 -/- B10 -/- B5 -/- Pool D4 -/- E6 -/-
D
Hep3B
E
HepG2
WT Pool H8 +/+ B4 -/- A2 -/- B5 -/- Pool D6 +/+ E2 -/- B6 -/- F2 -/-
20% O2
0.2% O2

Figure 3

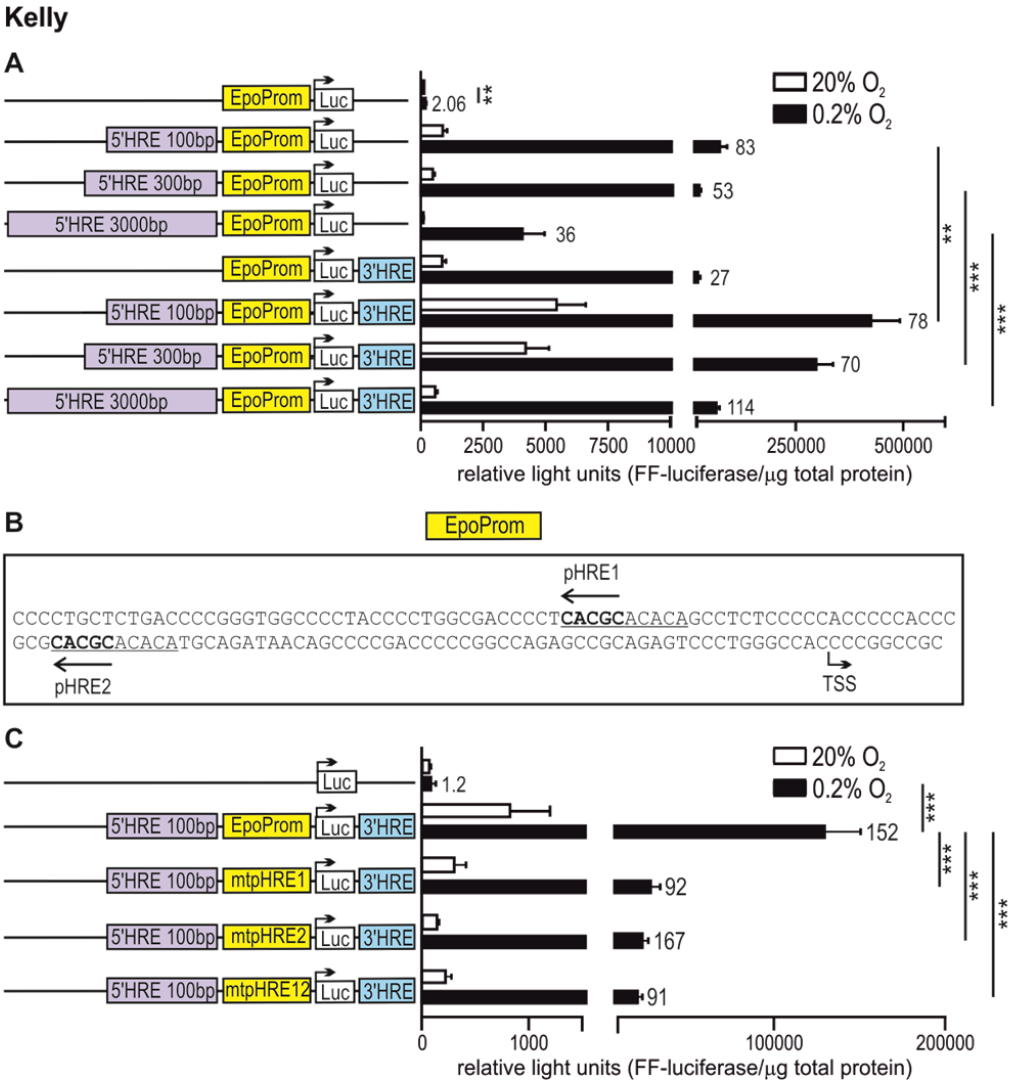

Kelly
A
EpoProm
Luc
3'HRE
5'HRE 100bp
5'HRE 300bp
5'HRE 3000bp
2.06
83
53
36
27
78
70
114
20% O2
0.2% O2
0
2500
5000
7500
10000
250000
500000
relative light units (FF-luciferase/µg total protein)
B
EpoProm
pHRE1
CCCCTGCTCTGACCCCGGGTGGCCCCTACCCCTGGCGACCCCTCACGCACACAGCCTCTCCCCCACCCCCACCC
GCGCACGCACACATGCAGATAACAGCCCCGACCCCCGGCCAGAGCCGCAGAGTCCCTGGGCCACCCCGGCCGC
pHRE2
TSS
C
mtpHRE1
mtpHRE2
mtpHRE12
1.2
152
92
167
91
0
1000
100000
200000
relative light units (FF-luciferase/µg total protein)

Figure 4

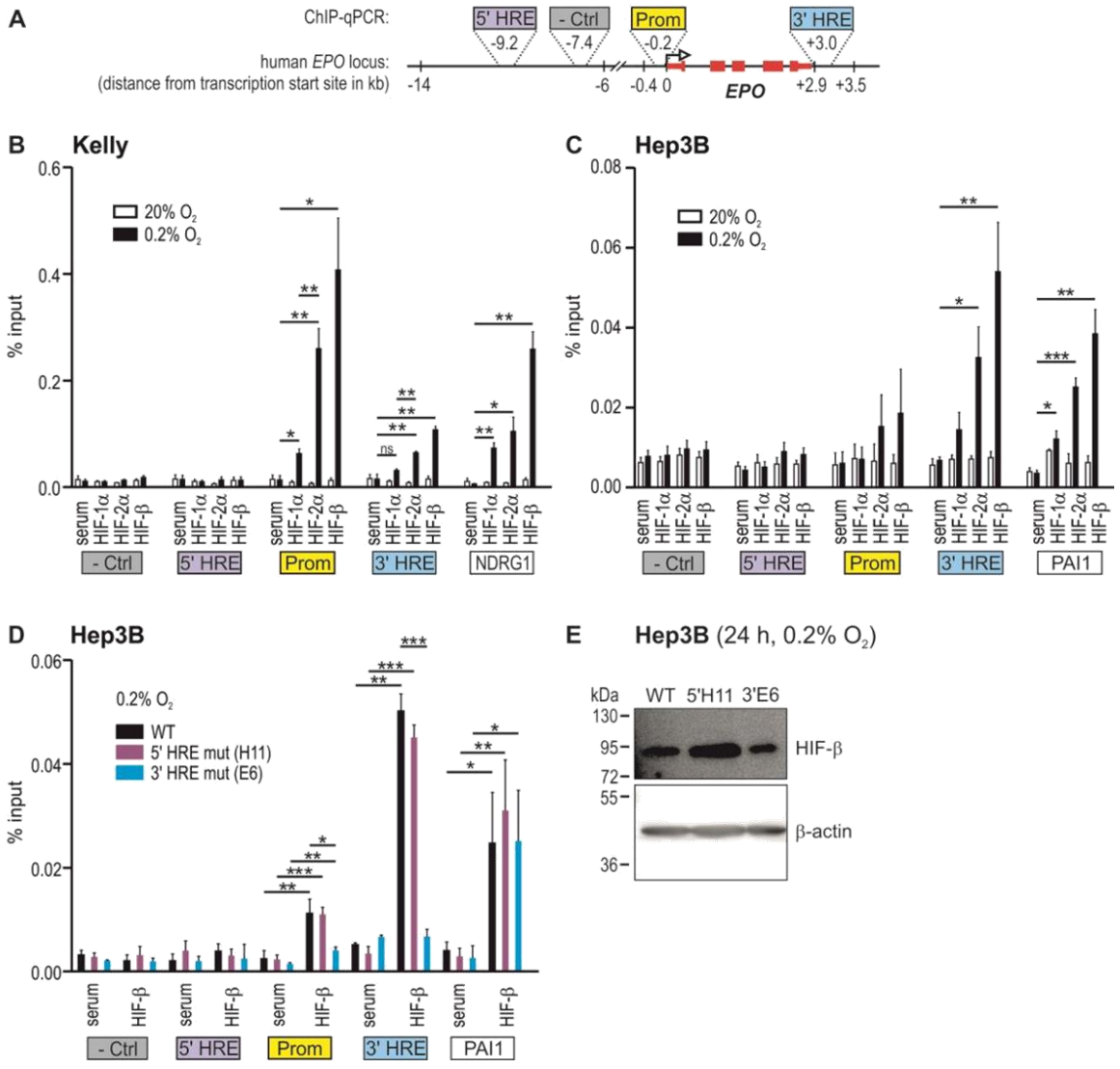
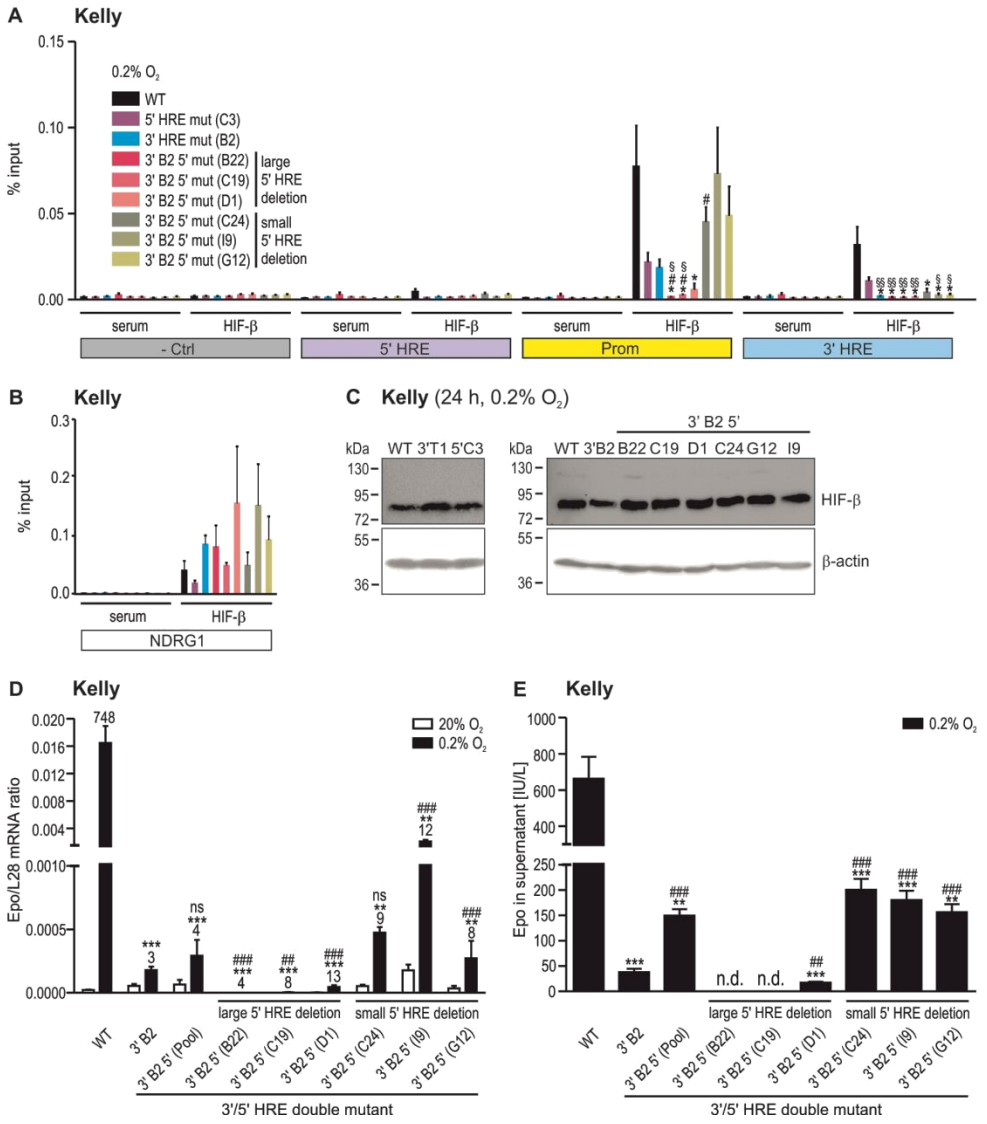

A
ChIP-qPCR:
5' HRE
- Ctrl
Prom
3' HRE
-9.2
-7.4
-0.2
+3.0
human EPO locus:
(distance from transcription start site in kb)
-14
-6
-0.4 0
EPO
+2.9 +3.5
B Kelly
C Hep3B
20% O2
0.2% O2
% input
serum
HIF-1α
HIF-2α
HIF-β
- Ctrl
5' HRE
Prom
3' HRE
NDRG1
PAI1
D Hep3B
0.2% O2
WT
5' HRE mut (H11)
3' HRE mut (E6)
E Hep3B (24 h, 0.2% O2)
kDa
WT 5'H11 3'E6
130
95
72
55
36
HIF-β
β-actin

Figure 5


A Kelly
0.2% O2
WT
5' HRE mut (C3)
3' HRE mut (B2)
3' B2 5' mut (B22)
3' B2 5' mut (C19)
3' B2 5' mut (D1)
3' B2 5' mut (C24)
3' B2 5' mut (I9)
3' B2 5' mut (G12)
large 5' HRE deletion
small 5' HRE deletion
% input
serum
HIF-β
- Ctrl
5' HRE
Prom
3' HRE
B Kelly
NDRG1
C Kelly (24 h, 0.2% O2)
3' B2 5'
kDa WT 3'T1 5'C3
kDa WT 3'B2 B22 C19 D1 C24 G12 I9
HIF-β
β-actin
D Kelly
20% O2
0.2% O2
Epo/L28 mRNA ratio
large 5' HRE deletion
small 5' HRE deletion
3'/5' HRE double mutant
E Kelly
Epo in supernatant [IU/L]
n.d.
3'/5' HRE double mutant

Supplementary Table 1. Oligonucleotide sequences.

sgRNA construction oligonucleotides (only sense strands shown) 5'hre-sgrna	5'-ccctgcacgtatgtgctccgggc-3'	
3'hre-sgrna	5'-ccctacgtgctgtctcacacagc-3'	
EPO HRE PCR amplification primers		
5'hre-fwd	5'-tggctgcggacatttctatc-3'	
5'hre-rev	5'-gatgcaggagcctggttcac-3'	
5'hre-rev (deep seq.)	5'-ttcagtggcaatgtggaggt-3'	
3'hre-fwd	5'-gcacctaccatcagggacag-3'	
3'hre-rev	5'-gccctgggcagggttg-3'	
EPO HRE PCR amplification/cloning primers		
5'hre-ecorv-fwd	5'-ccccgatatctgcggacatttctatcaggc-3'	
5'hre-xhoi-rev	5'-ccccccctcgagttcagtggcaatgtggaggt-3'	
3'hre-ecorv-fwd	5'-ccccgatatcgcacctaccatcagggaca-3'	
3'hre-xhoi-rev	5'-ccccctcgagctgggcagggttggcag-3'	
mRNA RT-qPCR quantification primers		
epo-fwd	5'-tgggggtgcacgaatg-3'	
epo-rev	5'-tttggtgtctgggacagtga-3'	
caix-fwd	5'-gggtgtcatctggactgtgtt-3'	
caix-rev	5'-cttctgtgctgccttctcatc-3'	
l28-fwd	5'-gcaattccttccgctacaac-3'	
l28-rev	5'-tgttcttgcggatcatgtgt-3'	
loxl2-fwd	5'-gagttgcctgctcagaaacc-3'	
loxl2-rev	5'-ctgtgacagtcgtgccagat-3'	
pai1-fwd	5'-actggaaaggcaacatgacc-3'	
pai1-rev	5'-gaggaagggtctgtccatga-3'	
ChIP qPCR quantification primers		Used for:
EPO-5'HRE-fwd	5'-gatggagctgtgtctccctg-3'	Figure 4
EPO-5'HRE-rev	5'-agtggcaatgtggaggtctg-3'	Figure 4
EPO-5'HRE-fwd	5'-ggctgcggacatttctatca-3'	Figure 5
EPO-5'HRE-rev	5'-aaggaagccttgcagtcag-3'	Figure 5
EPO-negctrl-fwd	5'-ggtgcttggtcaggagttga-3'	
EPO-negctrl-rev	5'-gacacgttctcgtccaacct-3'	
EPO-promoter-fwd	5'-gtcctgcccctgctctgacc-3'	
EPO-promoter-rev	5'-gctgttatctgcatgtgtgcgt-3'	
EPO-3'HRE-fwd	5'-cagcagtgcagcaggtccaggtcc-3'	Figure 4
EPO-3'HRE-rev	5'-cgagaggtcagacaggctgtgtgag-3'	Figure 4
EPO-3'HRE-fwd	5'-cttcaacctcattgacaagaactg-3'	Figure 5
EPO-3'HRE-rev	5'-ggatttggaggttcccagaaa-3'	Figure 5
NDRG1-HRE-fwd	5'-tccctcccaatctctctcttctt-3'	
NDRG1-HRE-rev	5'-caccatcagcacagcaaactac-3'	
PAI1-HRE-fwd	5'-cagagggcagaaaggtcaag3'	
PAI1-HRE-rev	5'-ctctgggagtccgtctgaac-3'	

Supplementary Methods

Gene editing

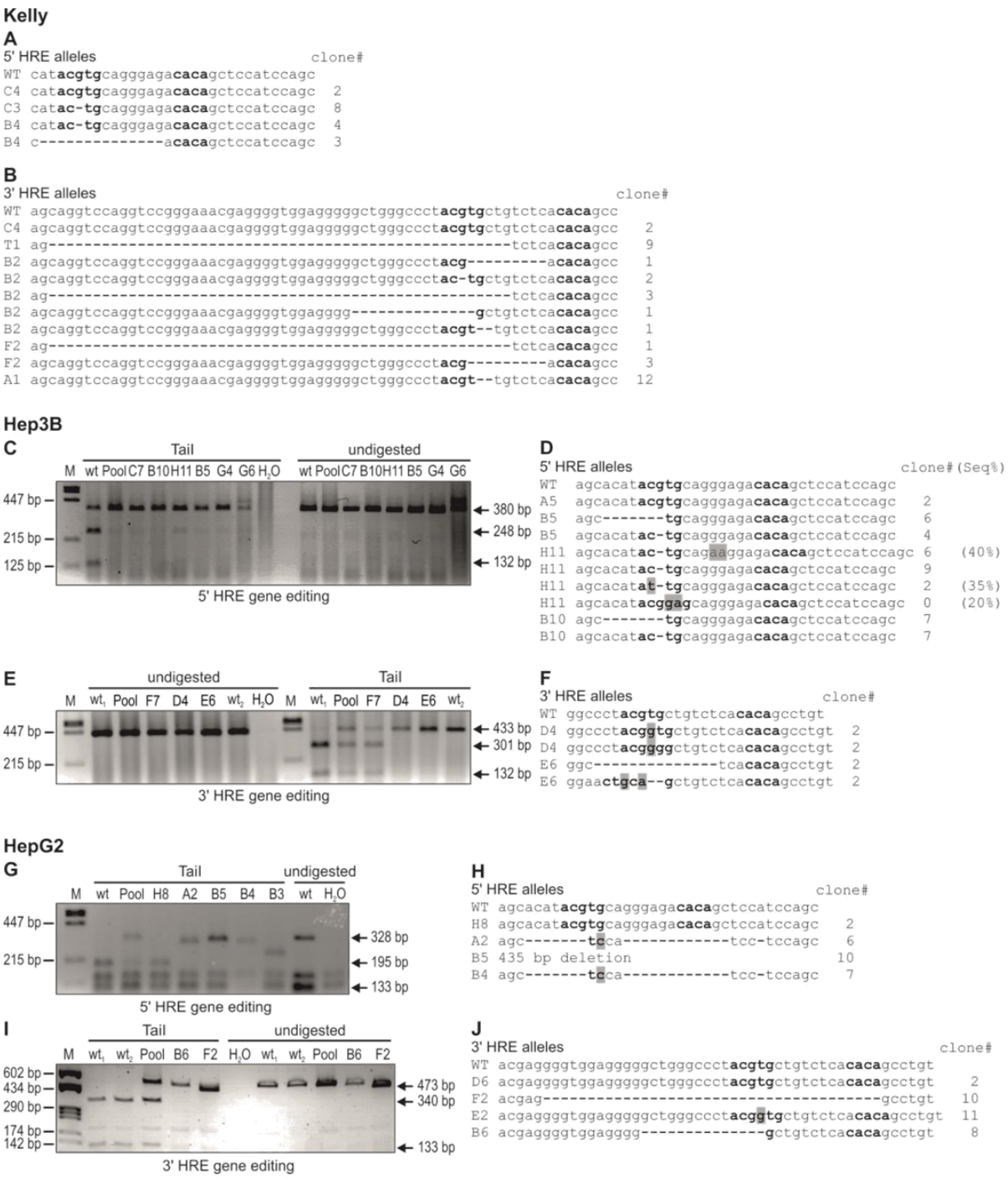
Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) gene editing was performed as described.(1) The EPO locus was screened for single guide RNA (sgRNA) binding sites using CRISPR Design Tool. (2) Oligonucleotides containing the sgRNA sequences (Supplementary Table 1) were synthesized (Microsynth, Balgach, Switzerland), annealed and ligated into plasmid MLM3636 (#43860; Addgene, Middlesex, UK). Cells were co-transfected with expression vectors for sgRNA, Cas-9 (ToolGen, Seoul, South Korea) and puromycin resistance (linearized pBabe vector; #1764; Addgene) using polyethylenimine (Polysciences, Warrington, PA, USA) for Hep3B and HepG2, and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for Kelly cells. Puromycin (1.5 µg/ml, Hep3B; 1 µg/ml, HepG2; 0.2 µg/ml, Kelly) was added two days after transfection for up to two weeks. Gene edited cells were cloned by limiting dilution and genotyped by PCR using High Fidelity Phusion Polymerase (Thermo Fisher Scientific) and the primers listed in Supplementary Table 1. Amplicons were analyzed either by TaiI digestion (Thermo Fisher Scientific) followed by 2% agarose gel electrophoresis to check for HRE destruction, (3) or by sub-cloning into pBluescript vector (Agilent, Santa Clara, CA, USA) and plasmid DNA sequencing (Microsynth), or by direct DNA deep sequencing (Functional Genomics Center Zurich).

References

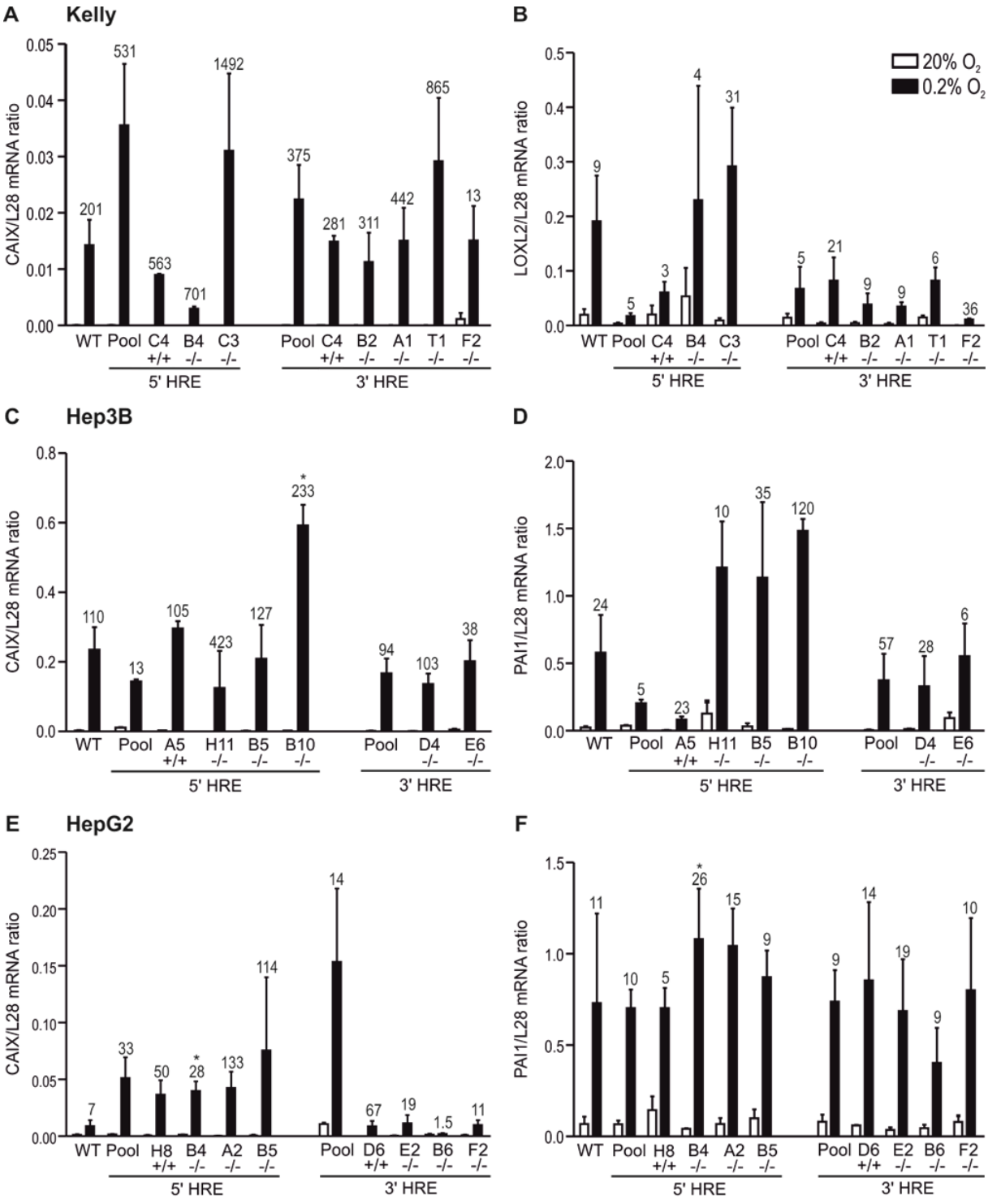
1. Harms DW, Quadros RM, Seruggia D, et al. Mouse genome editing using the CRISPR/Cas system. Current protocols in human genetics. 2014;83:15.17.11-27.

2. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature protocols. 2013;8(11):2281-2308.

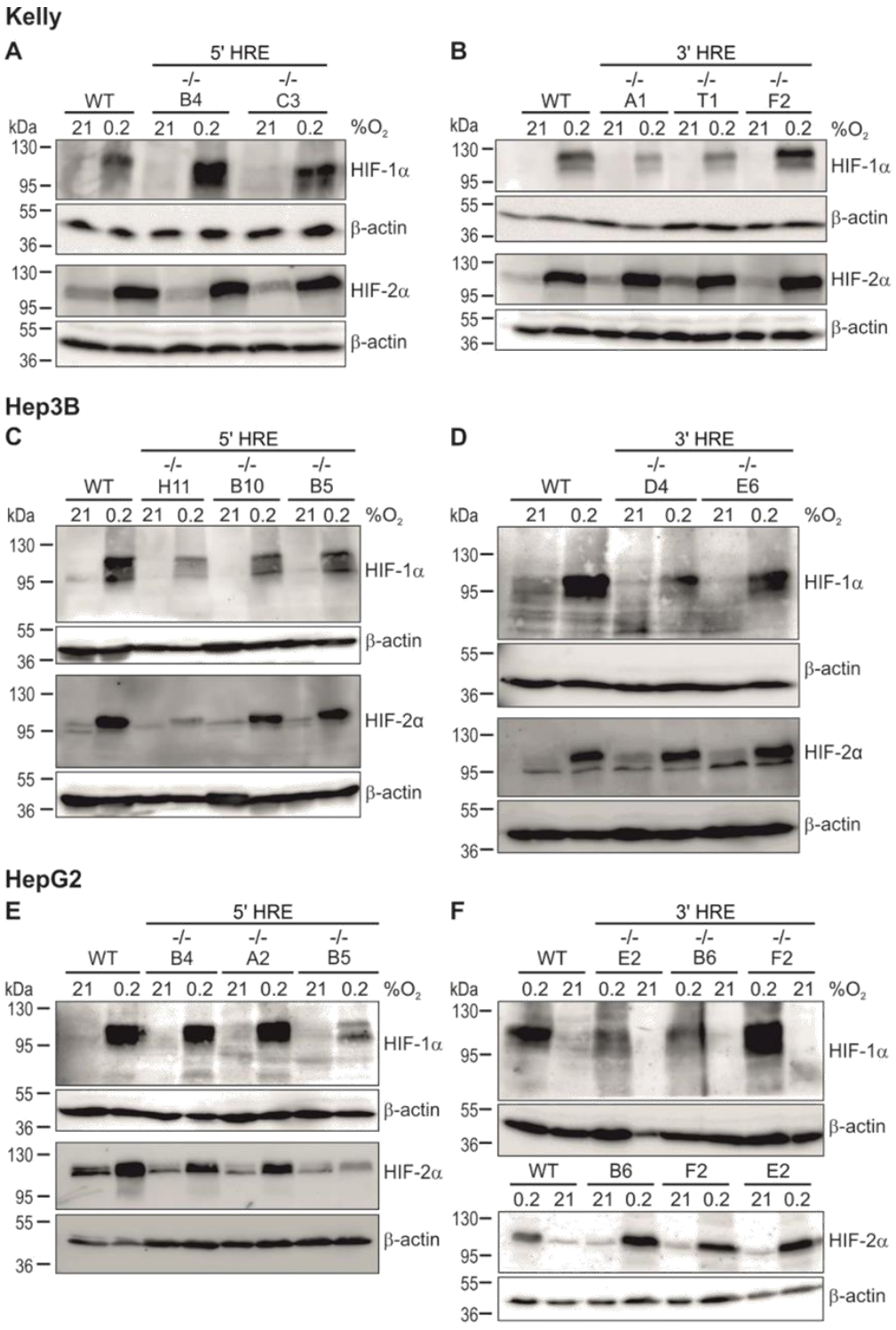
3. Wenger RH, Kvietikova I, Rolfs A, Camenisch G, Gassmann M. Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site. Eur J Biochem. 1998;253(3):771-777.



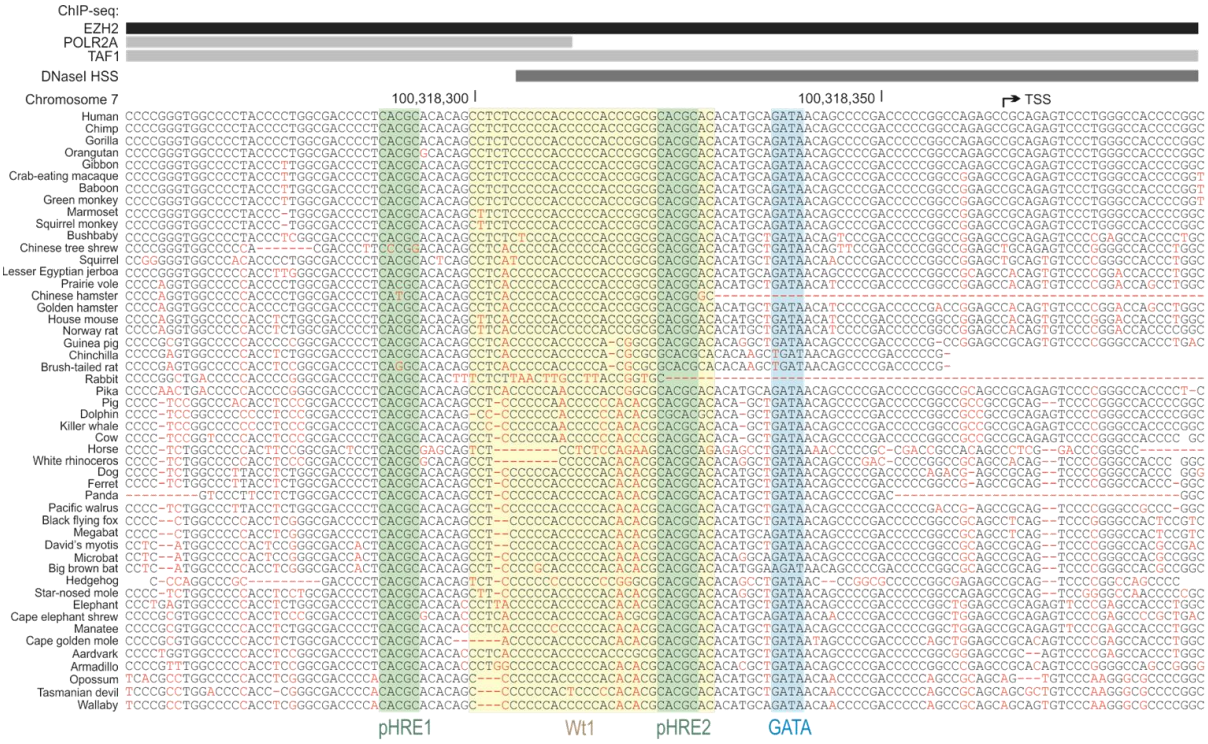
Supplementary Figure 1. Generation and analysis of *EPO* 5' and 3' mutant HRE clonal cell lines. Following mutation of the *EPO* 5' and 3' HREs by CRISPR-Cas9, Kelly (**A**, **B**), Hep3B (**D**, **F**) and HepG2 (**H**, **J**) cells were cloned by limiting dilution, the HRE regions amplified by PCR, and the amplicons either subcloned or directly deep sequenced (bold, consensus 5'-ACGTG-3' and 5'-CACA-3' HRE core and ancillary elements, respectively; WT, wild-type; dashes, nucleotide deletions; highlighted, nucleotide exchanges). The number of independent plasmids harboring the same sequence (clone#) and the percentage of deep sequence reads (Seq%) are indicated on the right. Like outlined for Kelly cells in Figure 1, mutation of the consensus HIF binding site confers resistance to Tail restriction digestion as shown by agarose gel analysis of digested or undigested PCR products using as template genomic DNA isolated from Hep3B (**C**, **E**) and HepG2 (**G**, **I**) cells (M, marker; wt; wild-type; Pool, polyclonal pool of cells: H_2O, water control).



Supplementary Figure 2. Hypoxic induction of HIF target genes in *EPO* 5' and 3' mutant HRE clonal cell lines. Kelly (**A**, **B**), Hep3B (**C**, **D**) and HepG2 (**E**, **F**) samples shown in Figure 2 were analyzed for the mRNA levels of the HIF target genes carbonic anhydrase (CA) IX (**A**, **C**, **E**), lysyl oxidase like (LOXL) 2 (**B**) and plasminogen activator inhibitor (PAI) 1 (**D**, **F**) by RT-qPCR. Transcript levels were normalized to ribosomal protein L28 mRNA and shown as mean + SEM of 3 independent experiments. Numbers above the columns indicate hypoxic induction factors. Student's t-tests were used to statistically evaluate the difference to hypoxic wild-type (WT) cells (*, $p<0.05$; Pool, polyclonal pool of cells; +/+, subclones containing two wild-type alleles; -/-, subclones with biallelic HRE mutation). Note that neither the hypoxic CAIX nor LOXL2 or PAI1 mRNA levels were significantly reduced by *EPO* HRE mutations.



Supplementary Figure 3. Hypoxic stabilization of HIF-1α and HIF-2α in *EPO* 5' and 3' mutant HRE clonal cell lines. Kelly (**A**, **B**), Hep3B (**C**, **D**) and HepG2 (**E**, **F**) wild-type (WT) cells and 5' HRE (**A**, **C**, **E**) or 3' HRE (**B**, **D**, **F**) mutant subclones were exposed to hypoxia (0.2% O_2) for 24 hours followed by immunoblotting for HIF-1α and HIF-2α. β-Actin was used as loading and blotting control. Note that HIFα protein stabilization was not affected by *EPO* HRE mutations.



Supplementary Figure 4. Putative *EPO* promoter HREs. Location of two conserved potential promoter HREs (pHRE1 and pHRE2; green) close to the GATA (blue) and Wt1 (yellow) sites in the *EPO* proximal 5' region. Shown is a UCSC Genome Browser output (version *hg19*), including 161 transcription factor ChIP-sequencing (ChIP-seq) tracks derived from the ENCODE database (version 3), clusters of DNaseI hypersensitivity sites (HSS) from 125 cell types, and the transcriptional start site (TSS), with a closer view of the region in 50 vertebrates extracted using the 100-MULTIZ whole-genome multiple sequence alignment algorithm.

Kelly

A

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5' HRE alleles in 3' HRE mutant B2 background                    deletion  (Seq%)
WT  ctcaggcccggagcacatacgtgcagggagacacagctccatccagcttc
B22 ctcaggccc-------------------------------atccagcttc   31 bp    (76%)
B22 ctcaggcccggagcaca-----------------gctccatccagcttc    18 bp    (22%)
C19 ctcaggccc-------------------------------atccagcttc   31 bp    (72%)
C19 ctcaggcccggagcaca-----------------gctccatccagcttc    18 bp    (24%)
D1  ctcaggccc-------------------------------atccagcttc   31 bp    (85%)
C24 ctcaggcccggagcacatac-tgcagggagacacagctccatccagcttc     1 bp    (94%)
I9  ctcaggcccggagcacatac--gcagggagacacagctccatccagcttc     2 bp    (99%)
G12 ctcaggcccggagcacat---tgcagggagacacagctccatccagcttc     3 bp    (92%)
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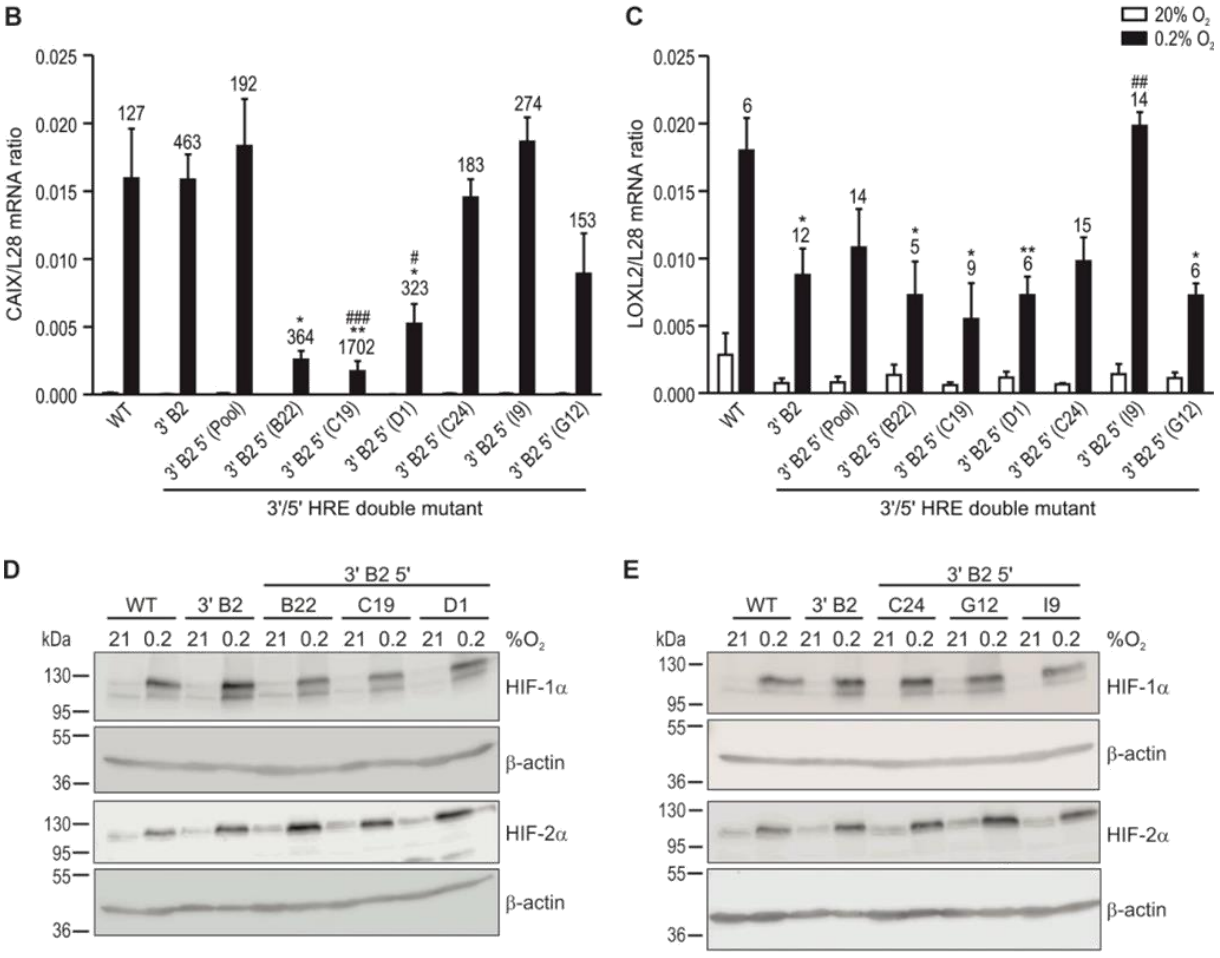

Supplementary Figure 5. Generation and analysis of *EPO* 5'/3' HRE double-mutant cell lines. (**A**) Following 5' HRE mutation of the Kelly 3' HRE mutant B2 subclone by CRISPR-Cas9 mediated gene editing, the resulting 5'/3' HRE double-mutant subclones were analyzed by PCR amplification and direct deep sequencing (bold, consensus 5'-ACGTG-3' and 5'-CACA-3' HRE core and ancillary elements, respectively; WT, wild-type; dashes, nucleotide deletions). The size of the HRE deletion and the percentage of deep sequence reads (Seq%) are indicated on the right. (**B, C**) Kelly samples shown in Figure 5D were analyzed for the mRNA levels of the HIF target genes carbonic anhydrase (CA) IX (**B**) and lysyl oxidase like (LOXL) 2 (**C**) by RT-qPCR. Transcript levels were normalized to ribosomal protein L28 mRNA and shown as mean + SEM of 3 independent experiments. Numbers above the columns indicate hypoxic induction factors. Student's t-tests were used to statistically evaluate the difference to hypoxic WT (*, $p<0.05$; **, $p<0.01$,) or 3' B2 cells ($^{\#}$, $p<0.05$; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$). (**D, E**) Kelly samples shown in Figure 5C were analyzed for the protein levels of HIF-1α and HIF-2α by immunoblotting. β-Actin was used as loading and blotting control.

4. Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation and a telocyte phenotype

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Running headline: Epo kinetics in renal telocyte-like cell lines

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Abstract

Erythropoietin (Epo) is essential for erythropoiesis and is mainly produced by the fetal liver and the adult kidney following hypoxic stimulation. Epo regulation is commonly studied in hepatoma cell lines, but differences in Epo regulation between kidney and liver limit the understanding of Epo dysregulation in polycythaemia and anaemia. To overcome this limit, we have generated a novel transgenic mouse model expressing Cre recombinase specifically in the active fraction of renal Epo-producing (REP) cells. Crossing with reporter mice confirmed the inducible and highly specific tagging of REP cells, located in the corticomedullary border region that displays a steep drop in oxygen bioavailability. A novel method was developed to selectively grow primary REP cells in culture and to generate immortalized clonal cell lines, called fibroblastoid atypical interstitial kidney (FAIK) cells. FAIK cells show a very early hypoxia-inducible factor (HIF)-2α induction which precedes Epo transcription. Like known for the kidney, Epo induction in FAIK cells reverses rapidly despite ongoing hypoxia, suggesting a cell autonomous feedback mechanism. In contrast, HIF stabilizing drugs resulted in chronic Epo induction. RNA-sequencing of three FAIK cell lines derived from independent kidneys revealed a high degree of overlap and suggests that REP cells represent a unique cell type with properties of pericytes, fibroblasts and neurons, known as telocytes. FAIK cells will be helpful to investigate myofibroblast differentiation in CKD and the molecular mechanisms of HIF stabilizing drugs currently in clincial phase III studies to replace Epo therapy in ESRD.

Introduction

Erythropoietin (Epo) regulated red blood cell homeostasis is crucial for oxygen delivery in vertebrates and Epo dysregulation causes anaemia or polycythaemia [1] [2]. While the liver is the main site of Epo production in the embryo, the kidney produces about 90% of circulating Epo in the adult and only the remaining 10% is of hepatic origin [3] [4]. Local tissue oxygenation appears to be the major factor triggering Epo production. Intriguingly, Epo is almost exclusively regulated on the mRNA level by hypoxia-inducible factor (HIF)-dependent trancriptional activation [5]. Whereas hepatic and neuronal cell lines are used to study Epo gene regulation, [6] [7] a reliable cell culture model derived from renal Epo-producing (REP) cells is currently not available.

Under normal conditions, REP cells are located in the peritubular interstitial space of the inner cortex [8]. A number of attempts to generate REP cell models have been reported. Cell lines derived from renal cell carcinoma occasionally produce Epo but oxygen-regulated Epo expression has not been found in these cancer cells [9]. Transgenic mice bearing a SV40

large T antigen in the *Epo* locus principally allowed for the isolation and immortalization of REP cells, but cultured cells showed no inducible Epo or large T expression [10]. Kidney-derived mesenchymal progenitor cells were differentiated *in vitro* to produce cell lines capable of oxygen-regulated Epo expression [11]. Transgenic mouse lines expressing fluorescent proteins under the control of regulatory elements derived from the *Epo* locus (knock-in allele in a background of severe neonatal anaemia) or from the *Col1a1* locus were used to isolate primary REP cells by flow cytometry [12] [13]. Human mesenchymal-like $CD133^{+}/CD73^{+}$ progenitor cells isolated from the inner medulla showed increased Epo production following hypoxic stimulation [14]. Finally, several conditional knock-out mouse models with constitutive HIF (over-)expression resulted in ectopic renal Epo synthesis, including models that involve the promoters derived from the genes encoding for CD68, renin, connexin 40, PDGFRβ and FOXD1 to drive Cre expression [15] [16] [17] [18] [19] [20] [21].

In conclusion, none of these attempts resulted in a reliable renal cell culture with permanent capability of regulated Epo expression, maybe due to the transient nature of *Epo* locus activation by differentiation and oxygen-dependent signals. We hence reasoned that isolation of freshly isolated cells acutely tagged for an active *Epo* locus may enhance the chance of obtaining REP cells. Therefore, we generated novel transgenic mouse lines to conditionally tag and isolate REP cells.

Results

Specific conditional targeting of active REP cells *in vivo*

A Cre^{ERT2} expression vector was constructed using 220 kb of the mouse *Epo* locus as shown in Figure S1. This construct was first tested *in vitro* to confirm hypoxia-inducible Cre expression and tamoxifen-dependent Cre activation (Figure 1A). Several transgenic founder lines were then generated and crossed with reporter mice which allowed for the permanent tagging of REP cells by red fluorescent tdTomato expression. While under normoxic conditions no or only very few fluorescent cells could be observed in the absence or presence, respectively, of tamoxifen (data not shown), this number increased following exposure to hypoxic conditions (Figure 1B). Pixel area quantification demonstrated that hypoxia clearly activated more REP cells in founder lines #1, #240 and #241 than in #244 (Figure 1C). *Epo-Cre^{ERT2}* #1 and #241 were chosen for subsequent analyses. As depicted in Figure 1D, tdTomato expression overlaps with immunoreactivity of CD73, an established marker of REP cells [22]. Furthermore, the CD73 surface marker revealed the long processes typical for REP cells [8]. High resolution microscopy showed in more detail the

irregular shape and the long processes of REP cells, extending between multiple tubular cells (Figure 1E), which further confirms the reported phenotype [8].

Whole-genome DNA sequencing revealed that both strains show single integration sites, but whereas the vector integrated in strain #1 into an intergenic region of chromosome 14 with the loss of only 330 bp, in strain #241 it integrated into chromosome 9 with the loss of 52.2 kb, including exons 4 to 13 of the *Pou2f3* gene. The remaining *Epo* flanking regions in the integrated BAC vectors were 45.9 kb and 98.2 kb in #1, and 75.2 kb and 52 kb in #241, upstream and downstream, respectively, of the Epo-coding region (Figure S2A). Both, the 5' and 3' boundaries of the integrated vectors were confirmed by PCR (examples shown in Figure S2B). Although the gross phenotypes of both founder strains were indistinguishable from wild-type mice, and despite identical REP cell tagging by tdTomato expression, strain #1 was chosen for further analyses due to the gene-disrupting nature of the vector integration into strain #241, which potentially could have deleterious effects [23].

REP cells localize to areas with abrupt decline in O_2-bioavailability

Renal Epo expression is exceptionally hypoxia-sensitive even though the HIF system and its hundreds of target genes are ubiquitously expressed [5]. While the underlying molecular mechanisms remain to be elucidated, the unique oxygen distribution in the adult kidney is likely involved in REP cell activation. Indeed, mice ubiquitously expressing a fusion protein between the HIF-1α oxygen-dependent degradation (ODD) domain and the luciferase (Luc) reporter gene showed a strikingly kidney-specific bioluminescence, as reported previously [24]. Only distal extremities and the normally hypoxic testis but no other inner organs showed a comparable hypoxia-induced bioluminescence (Figure 2A). Because whole-body imaging cannot reveal the intra-organ ODD-Luc distribution, we analyzed fresh organ slices of the kidneys shown in Figure 2A. Despite the now uniform tissue thickness, bioluminescence intensity in the medulla was still much stronger than in the cortex. Both, medulla and cortex displayed a quite homogenous bioluminescence distribution, with a steep O_2-bioavailability gradient mainly located in the corticomedullary border region (Figure 2B).

We next analysed the localization of REP cells in *Epo-Cre^{ERT2}*#1x*tdTomato* mice. In order to compare the delayed reporter protein accumulation with the short-lived Epo mRNA levels, we used two relatively strong hypoxic stimuli (each 4 hours of 0.1% CO inspiration) with one week recovery inbetween. While the first stimulus was used to permanently tag REP cells, the subsequent second stimulus was used to analyze acutely induced Epo mRNA. Both, tdTomato (Figure 2C) as well as endogenous Epo mRNA (Figure 2D) localized to peritubular interstitial cells of the corticomedullary border region. Strikingly, a much higher number of REP cells was observed by histochemical Epo mRNA *in situ* hybridization than tdTomato immunofluorescence, most likely due to the single-molecule sensitivity of the former method

which detects Epo mRNA levels far below the corresponding Cre expression threshold required for recombination of the *tdTomato* reporter locus. Therefore, we analysed Epo mRNA by the same technique following a more physiological hypoxic stimulus (3 hours of 8% O_2 inspiration). As shown in Figure 2E, this treatment results in a REP cell pattern similar to the tdTomato-tagged REP cells shown in Figure 2C. While no red cells were detected in wild-type (wt) mice, only sporadic cells were seen in transgenic mice following tamoxifen treatment. This number did not further increase after 4 hours of 8% O_2 inspiration (Figure 2F), suggesting that this stimulus is sufficient for Epo mRNA induction but borderline for functional Cre expression. However, there was a clear increase in REP cell numbers following 16 hours of 8% O_2 inspiration, demonstrating that this mouse model faithfully recapitulates the spatio-temporal conditional regulation of the *Epo* gene.

Isolation of primary REP cells by negative selection

Because the results shown above revealed that only a very small fraction of all cells of the kidney are active "on" REP cells, we reasoned that selection against Epo negative cells may be a more suitable strategy to isolate primary REP cells than selection for Epo positive cells. Therefore, *Epo-Cre*^{ERT2}#1x*tdTomato* reporter mice were crossed with the *Terminator* strain which allowed for the killing of non-Cre expressing cells using diphtheria toxin [25] (Figure S1). To select the acutely Epo expressing cells, freshly isolated renal cell suspensions were immediately exposed to hypoxia and tamoxifen to induce Cre-mediated recombination, followed by treatment with diphtheria toxin. While only few red cells were visible after 1.5 days of isolation, the abundance of tdTomato positive REP cells increased after 7 days of normoxic cultivation (Figure 3A). Most but not all of the viable cells were tdTomato positive after 10.5 days, suggesting that some cells may not have recombined the *tdTomato* allele. Epo mRNA was induced by hypoxia in primary REP cells after 6 days of isolation (Figure 3B), demonstrating that these proliferating primary REP cell cultures maintained the capacity to express and regulate the *Epo* gene.

Oxygen, DNA methylation and HIF-2 mediated Epo regulation in REP cell-derived FAIK cell lines

Because primary REP cells ceased to proliferate and lost inducible Epo expression after approx. 12-14 days of culture (data not shown), we generated permanent REP cell lines. Therefore, independent primary REP cell preparations were immortalized with SV40 large T antigen 3 or 6 days after isolation. Limited dilution cloning resulted in fibroblastoid atypical interstitial kidney (FAIK) cell lines. Three independent clones were chosen for further analysis. FAIK cells maintained hypoxia-inducible Epo protein for at least 30 passages (Figures 3C and S3).

Since the Epo signal intensity was rather weak we reasoned that during propagation some of the cells may have lost Epo expression due to epigenetic silencing by DNA methylation of Epo-regulatory loci or the *Epo* locus itself. DNA methyltransferase inhibition may hence restore Epo expression in these cells as recently shown in fibrotic kidneys *in vivo* [13]. Indeed, 5-azacytidine could enhance both basal and hypoxia-inducible Epo protein levels in FAIK3-5 cells (Figure 3C). These results were confirmed by ELISA using supernatants as well as cell lysates derived from the same cells (Figure 3D). A typical well-known feature of Epo gene expression is the early decrease after an initial strong induction, despite ongoing hypoxia and long before any change in haematocrit occurs [26] [27]. Interestingly, this feature could also be seen in FAIK cell lines as exemplified by Epo immunoblot detection in FAIK3-5 cells independent of whether 1 or 5 passages went by since the 5-azacytidine treatment (Figure 3E). Bisulfite sequencing of the *Epo* promoter region revealed mostly methylation-free proximal CpG dinucleotides whereas the more distal CpGs remained mostly methylated, independent of hypoxia or 5-azacytidine treatments (Figure 3F), suggesting that increased Epo expression following 5-azacytidine treatment is not due to direct action on the *Epo* promoter in FAIK cells.

mRNA levels in FAIK3-5 cells could efficiently be blocked using lentiviral transduction with shRNA constructs (Figure 3G and Supplementary Methods and corresponding Figures). shHIF-2α but not shCtrl or shHIF-1α blunted Epo expression, confirming HIF-2 dependent Epo regulation.

Faithful recapitulation of hypoxic Epo expression kinetics in FAIK cell lines

A well-established difference between HIF-1α and HIF-2α is the delayed (after 2 to 3 days) and prolonged hypoxic induction of HIF-2α protein often observed in cancer cell lines [28]. However, such a delayed HIF-2α induction would not be consistent with the rapid induction of its target gene *Epo* in REP cells *in vivo* and FAIK cell lines *in vitro*. Intriguingly, while HIF-1α showed maximal protein levels after 12 hours, HIF-2α induction was even faster in REP cells, reaching maximal levels after 3 to 6 hours of hypoxia (Figure 4A). Because such rapid kinetics is dominated by the slow oxygen diffusion and exchange rates [29] these experiments were repeated using cell culture media that had been pre-quilibrated with 0.2% O_2. While HIF-2α was already induced at the earliest time point (1.5 hours), the phosphorylated high molecular weight form of HIF-1α maximally accumulated after 12 hours (Figure 4B, upper panel). Under these conditions, Epo protein followed the HIF-2α kinetics with a maximal level already after 1.5 hours followed by a gradual decrease, as shown by immunoblotting (Figure 4B, lower panel), ELISA (Figure 4C) and mRNA quantification (Figure 4D). Of note, this rapid reversal of hypoxic Epo induction could not be observed using the PHD inhibitor FG-4592 (roxadustat), which led to a permanent Epo expression until

the end of the observation period after 48 hours (Figure 4E). As shown in Figure 4F, a single bolus of FG-4592 maintained Epo mRNA and protein levels for up to 72 hours, suggesting that chemical PHD inhibition blocks a potential negative feedback loop seen in prolonged hypoxia [30].

Transcriptomics of FAIK cell lines reveals metabolic adaptation to hypoxia

The three independent FAIK cell lines were further phenotyped by RNA sequencing (RNAseq) following exposure to hypoxia for 24 hours. Statistical evaluation confirmed the reproducibility of the REP cell isolation method (Figure S4A). Nevertheless, unsupervised cluster analysis showed that FAIK2-5 were more distant from FAIK1-10 and FAIK3-5, with normoxic and hypoxic FAIK2-5 being more closely related than the distinct normoxic and hypoxic clusters formed by the two other cell lines (Figure S4B). However, with a total of 16,357 expressed mRNA and lncRNA species, the majority (84.8%) was commonly expressed in all three cell lines. There was less overlap in the sequence read numbers of the hypoxically regulated (i.e. at least a 2-fold change) RNA species between the three cell lines, with 14.7% regulated, 21.1% induced and 8.8% repressed RNA species, respectively (Figure S4C). A rank order list of the 100 most strongly induced genes is shown in Figure S5. In addition to the approx. 700 to 950 mRNA species also 120 to 200 lncRNA species accumulated under hypoxic conditions in the three FAIK cell lines (Figure S4D), in line with our previous reports on hypoxic inducibility of lncRNAs [31][32]. Gene ontology analysis revealed metabolic reprogramming towards HIF-1 dependent glycolysis under hypoxic conditions (Table S1).

Established HIF target genes, [33] including VEGFa, Glut1, DEC2 and NDRG1, showed a very robust hypoxia-inducible expression, while housekeeping ribosomal protein rpL28 was constitutively expressed (Figure 5A). However, CAIX which is commonly induced in virtually all cancer cell lines, is almost completely absent even in hypoxic FAIK cells (data not shown), underlining the non-transformed nature of these cell lines. Of the mRNAs involved in the oxygen signalling cascade, PHD2 was most prominently expressed, in line with its predominant function in renal Epo regulation [34]. PHD2 and PHD3 but not PHD1 were significantly induced by hypoxia, consistent with a role in an intrinsic negative feedback loop [30]. Intriguingly in this context, also VHL (but not FIH) was induced by hypoxia, at least in the FAIK cell lines analysed (Figure 5B). Epo mRNA did not reach the threshold of 10 normalized sequence reads. Considering (i) the basal levelsclose to zero, (ii) the non-optimal time point for Epo mRNA induction, (iii) the small size of the Epo mRNA, and (iv) the low Epo versus housekeeping control ratio usually observed by reverse-transcription quantitative polymerase chain reaction, RNA sequencing was not sensitive enough for Epo detection under these experimental conditions. However, manual inspection revealed Epo mRNA

sequencing reads below the threshold in all 3 FAIK cell lines under hypoxic conditions only (data not shown).

FAIK cells resemble the telocyte subtype of pericytes/fibroblasts

To characterize the developmental origin of FAIK cell lines, a panel of cell-type specific markers was analysed. All FAIK cell lines were positive for the fibroblast markers PDGFRβ [19], CD73 [22] and Col1A1 [13] (Figure 5C). Also FSP1 [35] as well as the myofibroblast marker αSMA [36] were expressed, indicating that at least a subpopulation of these cells may have differentiated during cell culture.

REP cells have recently been shown to express the neuronal markers MAP2 and NFL [8]. While we could confirm the expression of MAP2, NFL was not found in FAIK cell lines, in line with a previous report [19]. However, they were clearly positive for the neuronal markers NGF, BDNF and nestin, as well as for the pericyte marker NG2/CSPG4 (Figure 5D) that has recently been used to trace brain pericytes as a major source of Epo in the mouse brain [37]. The neural crest markers P0 and Pax3, which have been used for the lineage tracing of REP cells, [38] [39] could either not be detected in FAIK cells or only at low levels (data not shown).

The transcription factors FoxD1 and WT1 are markers of the mesenchymal stroma precursor cells of the developing kidney which give rise to renal interstitial cells, including fibroblasts and pericytes. FoxD1 has previously been used to trace REP cells [20]. Both markers are expressed in all FAIK cell lines and WT1 was strongly induced by hypoxia. Because WT1 is known to regulate the *Epo* promoter [40], hypoxia-inducible WT1 may potentiate Epo induction in REP cells. The macrophage marker CD68 is weakly expressed in a hypoxia-inducible manner in all FAIK cell lines. Notably, CD68 promoter-driven Cre transgenic mice have previously been used to trace REP cells [15]. Furthermore, FAIK cells were also positive for the stemness marker CD24, also known as mouse heat-stable antigen [41], and the hematopoietic progenitor marker CD34 [42], although the latter could not be detected in FAIK2-5 cells (Figure 5E). However, endothelial cell marker CD31 as well as markers of leukocytes (CD45), dendritic cells (CD8α, CD11b, CD103) and macrophages (Mac1, F4/80) could not be detected, clearly demonstrating that FAIK cell lines are not of myeloid origin.

The morphology of primary REP cells as well as FAIK cell lines showed some characteristic features (e.g. few long and thin membrane protrusions partially forming cell-cell connections, large nuclei and an apparent lack of nucleoli in DAPI stainings) which are reminiscent of a subpopulation of pericytes/fibroblasts also referred to as "telocytes" [43]. Telocytes can be found in most organs, including in the kidney cortex (but not medulla) [44] [45] [46]. Telocytes display a number of phenotypical and transcriptomic properties that distinguish them from "classical" fibroblasts [43] [47] [48]. As shown in Table S2, there is an intriguing overlap

between markers expressed in telocytes and in FAIK cell lines. These markers were compiled from reports on telocytes derived from various organs, and some of the mismatches may hence be explained by the fact that only little information about telocytes isolated from the kidney is available.

FAIK cell lines kept in low density culture commonly form long and thin membrane protrusions (nanotubes), some of which apparently establish cell-cell connections (Figure 6A). These nanotubes can reach lengths of more than 100 µm and are positive for TOM20 (mitochondria), β-actin (microfilaments) and α-tubulin (microtubuli) (Figures 6B-D), typical for tunneling nanotubes [49]. Such mitochondria-containing nanotubes represent another hallmark of telocytes where they are referred to as "telopodes" [43]. Of note, while REP cells *in vivo* contain far less mitochondria than the neighbouring tubular epithelial cells, mitochondria could also be detected in the membrane protrusions of REP cells (Figure 6E). Finally, all three FAIK cells lines are positive for CD73 as well as for CD34 and PDGFRβ (Figure 6F), consistent with marker expression in telocytes [45][47].

Discussion

The renal Epo source has been discussed in the literature quite controversially over the past decades. Not only peritubular interstitial cells, [22] [50] [51] [52] but also proximal and distal convoluted tubules, intercalated cells of the collecting ducts and glomerular cells [53] [54] [55] [56] have been reported to contain Epo mRNA. These findings were apparently consistent with transgenic mouse models expressing reporter genes under the control of DNA fragments derived from the *Epo* locus [57][58] However, these data were all obtained by "classical" mRNA *in situ* hybridization or by using genomic *Epo* DNA fragments of limited length. With the recent refinement of the *in situ* hybridization technique, exclusively peritubular interstitial cells at the corticomedullary border were found to be Epo mRNA positive [59]. These findings have been confirmed by transgenic mouse models expressing reporter genes under the control of longer regions of the *Epo* locus,[10] [60] with approx. 200 kb long BACs most faithfully recapitulating the endogenous Epo expression pattern [8]. The results presented in our study confirm corticomedullary peritubular interstitial cells as sole REP cell type. The corticomedullary region showed the deepest drop in O_2-bioavailability which may expand into the cortex upon decreased tissue oxygenation, a prerequisite for the recruitment of additional REP cells [61].

Our animal model allowed for the conditional tagging of acutely recruited REP cells, whereas in previously established constitutively Cre-driven REP models it remained unknown whether labelled REP cells were still active [8] [39] [12] [62]. Whole-genome sequencing of two *Epo-Cre*^{ERT2} founder strains revealed that 45.9 kb 5' and 52 kb 3' flanking regions are sufficient to

confer accurate tissue-specific and oxygen-regulated *Epo* gene expression in the kidney. While Epo is known to be expressed in several other organs [2], we only occasionally detected tagged cells in liver and brain (data not shown). This may be explained by comparably low Epo levels per cell, but quite high overall Epo expression per organ, because a far higher percentage of liver (hepatic and stellate) and brain (neuronal, glial and pericytic) cells are (mildly) Epo positive compared to the proportion of (strongly) Epo positive cells in the kidney. Overall, these findings suggest that our novel conditional mouse model selectively labels only those cells with strong acute Epo transcription rates, ideally suited for the generation of primary active “on” REPcells.

While we did not obtain any detectable Epo expression in previously published REP cell lines [11] [63] (data not shown), the strong transcriptomic correlation of three independent REP-derived FAIK cell lines demonstrated the reproducibililty of the described procedure. More than 50% of the isolated FAIK clones still showed hypoxia-inducible Epo expression, but even these clonal cell lines apparently kept the typical REP cell property of the "on-off" behavior of *Epo* gene expression [64] [65]. This REP cell feature is poorly understood and may involve intrinsic transcriptional negative feedback loops as well as epigenetic mechanisms [13] [30]. Indeed, treatment with 5-azacytidine increased Epo levels, suggesting an at least partial epigenetic silencing in "off" REP cells by DNA methylation. Intriguingly, FG-4592/roxadustat, the most advanced PHD inhibitory drug currently in clinical phase III studies for the treatment of renal anaemia [66] led to a constitutive Epo induction in FAIK cells, whereas hypoxia only transiently induced Epo, suggesting differences in the negative feedback regulation and/or interference with myofibroblast differentiation [67]. However, our findings on HIF-2α/Epo kinetics clearly demonstrate that the transient *Epo* expression in chronic hypoxia is a cell autonomous effect and does not require e.g. altered blood composition or microenvironmental changes.

RNAseq of FAIK cells allowed for a thorough characterisation of the cellular identity of REP cells. Based on these data, we suggest that REP cells represent a unique subset of interstitial pericytes/fibroblasts which are also known as telocytes and which were found to express a number of neuronal markers. While further analyses will be required to confirm this suggestion, it is an intriguing idea that also telocytes residing in other organs may represent major sources of non-renal Epo. Of note, while both neurons and astrocytes have been well-established as Epo-producing cells of the brain [68] [69], a recent report demonstrated that brain pericytes actually represent a major source of cerebral Epo upon hypoxic stimulation [37]. It will be of interest to analyse whether Epo-producing pericytes of the brain also share features of telocytes.

Material and Methods

Animals

Epo-Cre^{ERT2} transgenic mice were generated according to previously published methods, [70] using a newly constructed expression vector as detailed in Supplementary Methods. All animal experiments were approved by the veterinary office of the canton Zurich (license numbers ZH126/13 and ZH233/15).

Tissue analyses

Mouse kidney sections were analyzed by immunofluorescence or *in situ* hybridization using RNAscope technology (see Supplementary Methods for antibody and imaging details).

Cell culture

Primary REP cells and clonal cell lines were generated and characterized as outlined in Supplementary Methods.

Protein and transcript analyses

Mouse Epo protein was detected by ELISA and immunoblotting as described previously [71] [72] mRNA levels were quantified by RT-qPCR as described previously [28] using the PCR primers provided in Table S3. Poly-A selected RNA was sequenced as detailed in Supplementary Methods and the RNAseq data were deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena; study accession number PRJEB19328).

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DISCLOSURES

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Supplementary Methods.
Figure S1. Generation of a mouse line to conditionally target renal Epo-producing (REP) cells and to select against non-REP cells.
Figure S2. Genotyping of *Epo-Cre*^{ERT2} transgenic mouse lines.
Figure S3. Persistent hypoxia-inducible Epo expression in FAIK3-5 cells.
Figure S4. RNAseq of FAIK cell lines.
Figure S5. Heat map of hypoxia-inducible gene expression in FAIK cell lines.
Figure S6. Testing of antibodies to detect mouse Epo and HIFα.
Table S1. List of significantly ($p < 0.05$) enriched KEGG pathways in the three FAIK cell lines. The input list contained all genes that were at least two-fold hypoxically induced ($p \leq 0.001$) as determined by RNAseq.
Table S2. Comparison of marker gene expression in telocytes andfibroblastoid atypical interstitial kidney (FAIK) cell lines.
Table S3. Primers used for polymerase chain reaction.
Supplementary information is available at *Kidney International's* website.

Figure 1

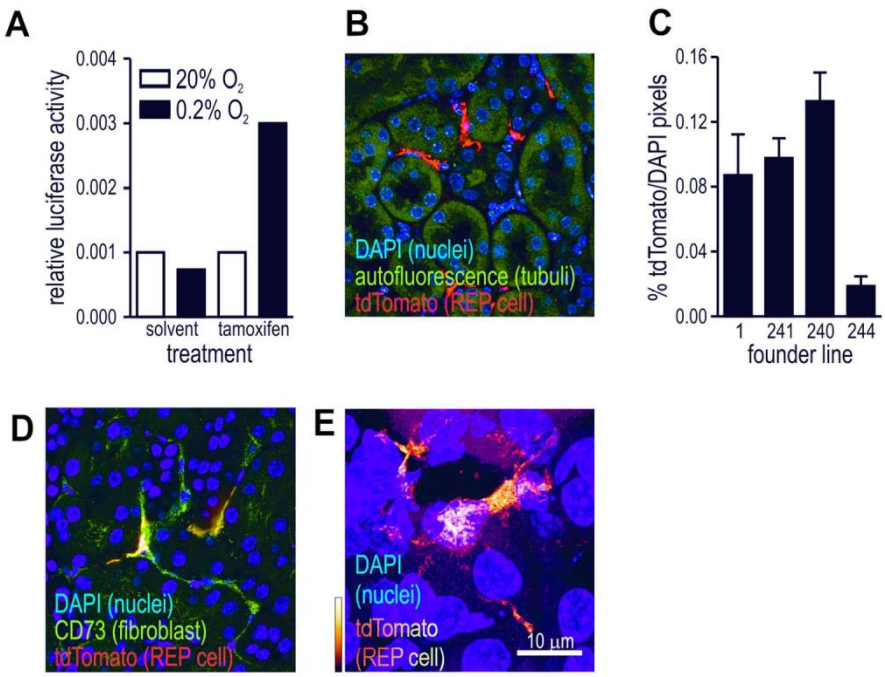

Figure 1. Conditional targeting of renal Epo-producing (REP) cells *in vivo*. (A) *In vitro* analysis of the *Epo-Cre*^{ERT2} BAC transgenic vector in Hep3B cells by co-transfection of the BAC construct together with Cre-inducible and constitutive reporter genes. Dual luciferase reporter gene assays were performed following exposure to tamoxifen and/or hypoxia (0.2% O_2 for 20 hours). (B) Red fluorescent tdTomato expression in REP cells of *Epo-Cre*^{ERT2}#241x*tdTomato* mice following exposure to tamoxifen and hypoxia (0.1% CO for 4 hours). Nuclei were stained with DAPI (blue) and tubuli were visualized by their autofluorescence (green). (C) REP cell labelling of the initial *Epo-Cre*^{ERT2} founder lines following crossing with tdTomato reporter mice and exposure to tamoxifen and hypoxia. tdTomato was detected using an anti-RFP antibody and 7 to 11 sections of two female mice (3 to 5 months old) for each founder line were scanned on a slidescanner. The pixel areas of tdTomato and DAPI fluorescence over a manually defined threshold were quantified and the tdTomato area is shown as percentage of the DAPI area (mean + SEM). (D) REP cell co-staining (yellow) by anti-CD73/ecto-5'-nucleotidase antibodies (green) together with tdTomato fluorescence (red) following exposure to tamoxifen and hypoxia (8% O_2 for 16 hours) of *Epo-Cre*^{ERT2}#1x*tdTomato* mice. Triple staining together with the DAPI nuclear stain (blue) results in white signals. (E) Deconvolution of laser scanning fluorescence microscopy of *Epo-Cre*^{ERT2}#241x*tdTomato* mice following exposure to tamoxifen and hypoxia (0.1% CO for 4 hours).

Figure 2

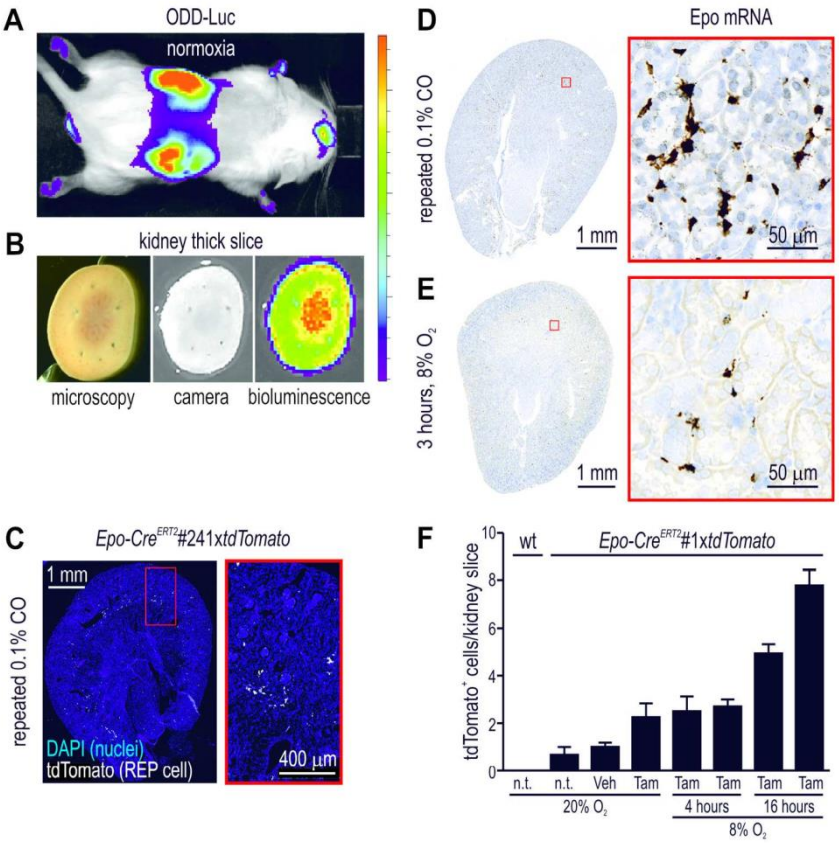

Figure 2. Hypoxic activation and localization of REP cells. (A) Bioluminescence *in vivo* imaging following s.c. injection of luciferin into a male mouse ubiquitously expressing the oxygen-labile ODD-Luc fusion protein. (B) Bioluminescence imaging of a transversal thick slice (500 µm) of the kidney shown in (A). Bright field microscopy (left), *in vivo* imaging system (IVIS) camera in bright light (middle), and IVIS bioluminescence (right) pictures are shown, demonstrating that the highest bioluminescence (red) is located in the medulla, medium bioluminescence (green) in the cortex, and lowest bioluminescence (blue) in the large vessels and outer cortical rim. (C) Immunofluorescence microscopy to localize tdTomato-positive REP cells detected using an anti-RFP antibody (white) in a kidney derived from a *Epo-Cre^{ERT2}*#241x*tdTomato* mouse after tamoxifen treatment (daily oral gavage of 5 mg tamoxifen for 5 consecutive days) followed by a repeated exposure to 0.1% CO for 4 hours with 1 week recovery inbetween. (D-E) Kidney Epo mRNA *in situ* hybridization of *Epo-Cre^{ERT2}*#241 mice exposed to CO as described above (D) or to inspiratory hypoxia (8% O_2) for 3 hours (E). (F) Relative numbers of tdTomato-positive REP cells per kidney slice in non-treated (n.t.) *Epo-Cre^{ERT2}*#1x*tdTomato* mice, or after vehicle (DMSO; Veh) or tamoxifen (Tmx) pre-treatment (daily oral gavage of 2 mg tamoxifen for 4 consecutive days) followed by exposure to inspiratory hypoxia as indicated. Each bar represents average values +SEM of 6-20 slices derived from one mouse as indicated. No red fluorescent cells were detected in wild-type (wt) control mice.

Figure 3

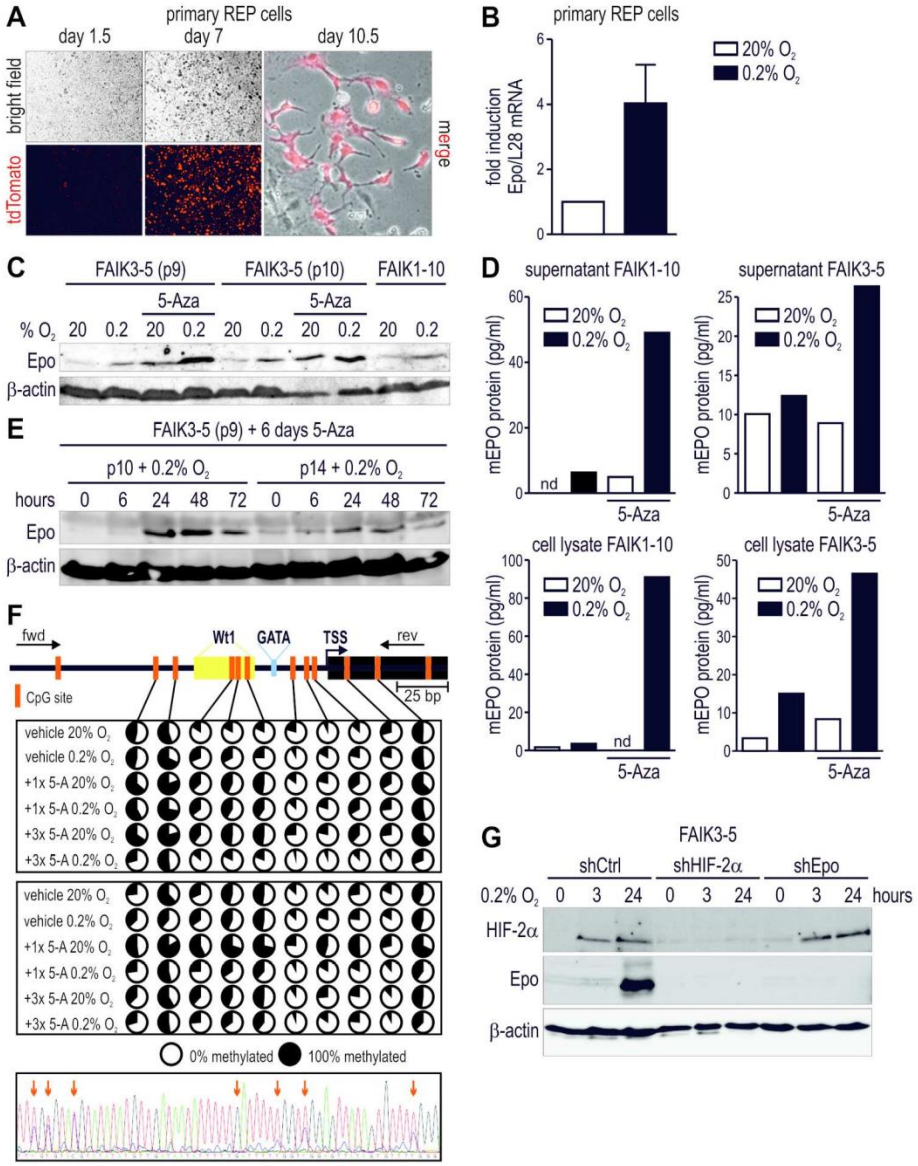

Figure 3. Generation and characterisation of FAIK cell lines derived from primary REP cells. (A) Bright field (upper panel) and tdTomato fluorescence (lower panel) microscopy of the same primary REP cell preparation 1.5, 7 and 10.5 days after isolation following 3 days of selection with diphtheria toxin. (B) Hypoxic Epo mRNA induction in primary REP cells 6 days after cell isolation by exposure to 0.2% O_2 for 24 hours. Ratios between Epo (measured by exon 4-5 RT-qPCR) and ribosomal protein L28 mRNA levels were normalized to the normoxic control of each individual experiment. Shown is the mean induction factor +SEM (n=6). (C) Immunoblot detection of Epo protein in FAIK cell lines. FAIK3-5 cells at passage 9 or 10 were either left untreated or were pre-incubated with 1 µM 5-azacytidine (5-Aza) for 6 days, passaged once more, and then exposed to hypoxia (0.2% O_2) for 24 hours. (D) ELISA measurements of mouse Epo (mEpo) protein in the supernatant (upper panel) and cell lysates (lower panel) of the same cells as shown in (C) (nd, not detectable). (E) Time course of hypoxic Epo protein induction in FAIK3-5 cells after pre-incubation with 5-Aza as in (C), followed by exposure to hypoxia for the indicated time periods 1 or 5 passages later. (F) CpG methylation of the *Epo* promoter region in FAIK1-10 cells determined by bisulfite sequencing. An example PCR product sequencing track is shown in the lower panel (red arrows, CpG sites). (G) Knock-down of HIF-2α or Epo by shRNA expression (shCtrl, unrelated control shRNA). β-Actin served as loading and blotting control in all immunoblots.

Figure 4

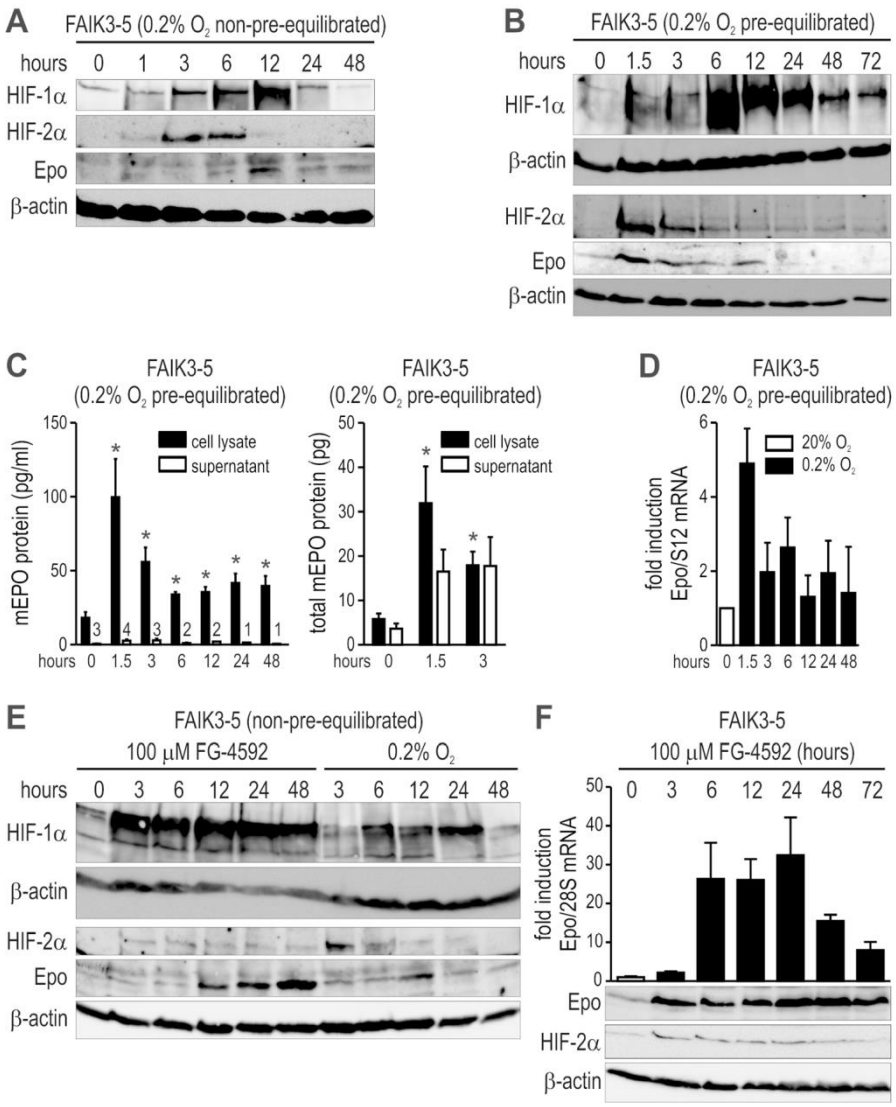

Figure 4. HIF and Epo expression kinetics. Time course of hypoxic HIF-1α, HIF-2α and Epo levels in FAIK3-5 cells following exposure to 0.2% O_2 in a conventional incubator (A) or after medium replacement by pre-equilibrated medium in a hypoxic workstation (B). (C) ELISA determination of Epo concentrations (left panel) and total amounts (right panel) in lysates and supernatants (n above background is indicated) prepared from cells treated as in (B). Shown are mean values + SEM of n=4 independent experiments. Hypoxic increase vs. normoxic control was evaluated using unpaired two-tailed T-tests (*, $p<0.05$). (D) Ratios between Epo (measured by exon 3/4-4 RT-qPCR) and ribosomal protein S12 mRNA of cells treated as in (B) (shown are mean hypoxic induction factors + SEM; n=3). (E) Time course of hypoxic HIF-1α, HIF-2α and Epo protein induction in FAIK3-5 cells following exposure to FG-4592 (roxadustat) or 0.2% O_2.

Figure 5

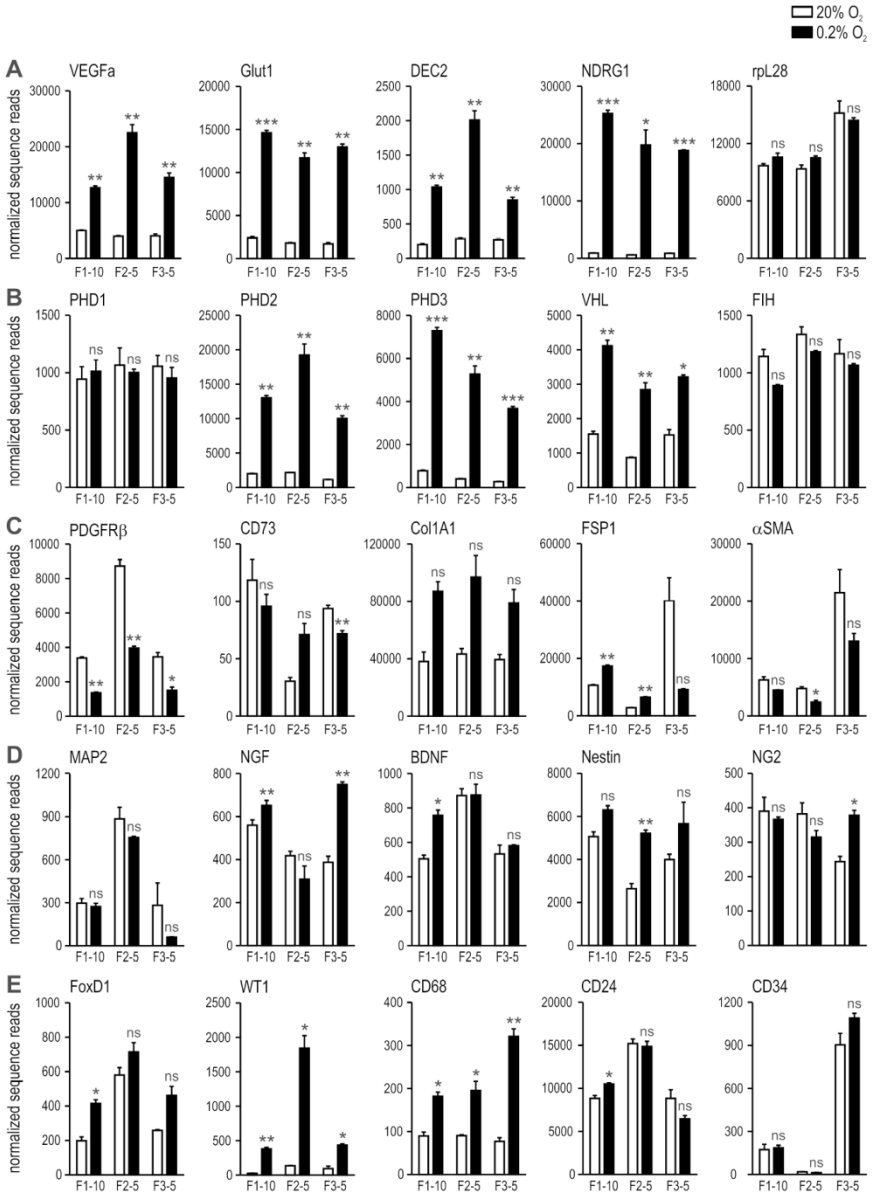

Figure 5. Oxygen-regulated and cell type specific marker gene expression in FAIK cell lines. Normalized RNA sequence read numbers in FAIK1-10 (F1-10), FAIK2-5 (F2-5) and FAIK3-5 (F3-5) cell lines determined by RNAseq as shown in Figures S4 and S5. (A) Transcript levels of established hypoxia-inducible and control genes. Vascular endothelial growth factor a, VEGFa; glucose transporter 1, Glut1/Slc2a1; differentially expressed in chondrocytes, Dec2/Bhlhe41/Sharp1; N-myc downstream regulated 1, NDRG1; ribosomal protein L28, rpL28. (B) Transcript levels of genes involved in the oxygen signalling cascade. Prolyl-4-hydroxylase domain (PHD) 1 to 3; von Hippel-Lindau, VHL; factor inhibiting HIF, FIH/HIF1AN. (C) Transcript levels of marker genes of the fibroblast lineage. Platelet-derived growth factor receptor β, PDGFRβ; cluster of differentiation (CD) 73, CD73/Nt5e; collagen 1A1, Col1A1; fibroblast-specific protein 1, FSP1/S100A4; α smooth muscle actin, αSMA/Acta2. (D) Transcript levels of marker genes of the neuronal lineage. Microtubule-associated protein 2, MAP2; nerve growth factor, NGF; brain-derived neurotrophic factor, BDNF; neuron-glial antigen 2, NG2/CSPG4. (E) Transcript levels of hematopoietic marker genes. Forkhead box D1, FoxD1; Wilms tumor protein 1, WT1; macrosialin, CD68; mouse heat-stable antigen, HSA/CD24; hematopoietic progenitor cell antigen, CD34. Shown are mean values + SEM of 3 independent experiments. Hypoxic regulation was evaluated using paired two-tailed T-tests (ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 6

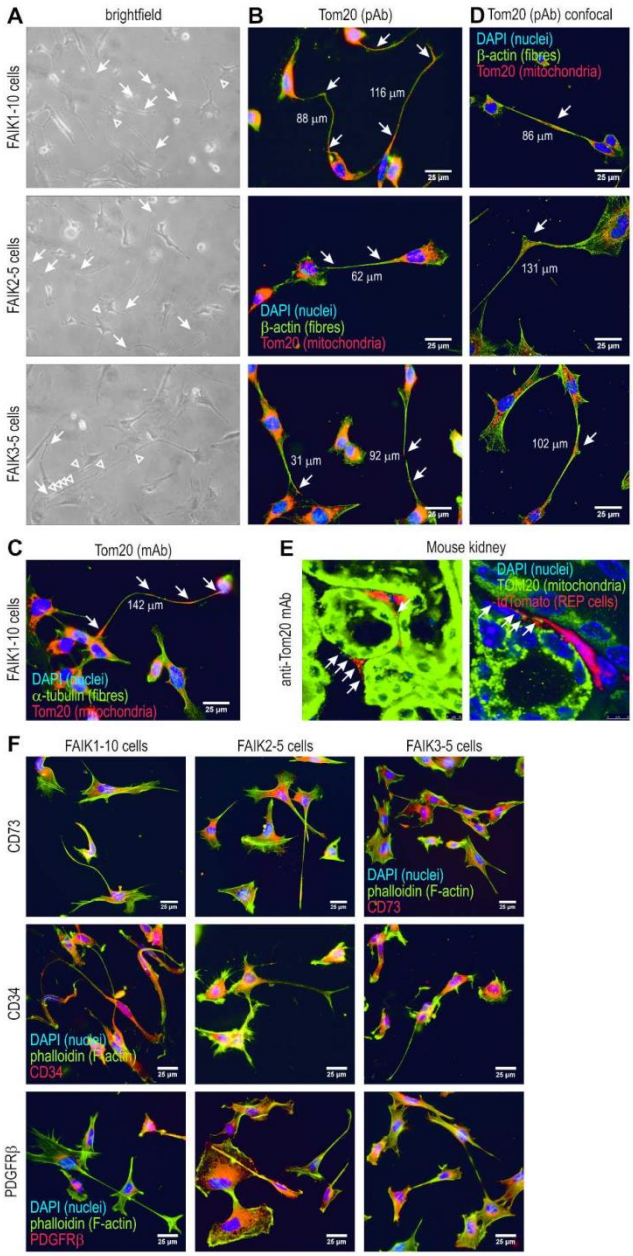

Figure 6. Telocyte phenotype of FAIK cell lines. (A) Live microscopy showing thin membrane protrusions (nanotubes) formed in low-density FAIK cell culture. (B-D) Localization of mitochondria in FAIK cell lines following immunodetection by polyclonal (pAb) or monoclonal (mAb) anti-TOM20 antibodies using epifluorescence (B,C) or confocal (D) microscopy in combination with anti-β-actin (B,D) or anti-α-tubulin (C) immunodetection. (A-D) Arrows, nanotubes containing mitochondria; arrow heads, nanotubes forming cell-cell contacts; tube lengths are indicated in µm. (E) TOM20 immunofluorescence of *Epo-Cre*^{ERT2}#1x*tdTomato* mouse kidney. Arrows indicate mitochondria in REP cells. (F) Immunofluorescence detection of the telocyte markers CD34 and PDGFRβ. β-actin was stained with phalloidin.

References

1. Semenza GL. Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. *Blood.* 2009;114:2015-2019.

2. Wenger RH, Kurtz A. Erythropoietin. *Compr Physiol.* 2011;1:1759-1794.

3. Schooley JC, Mahlmann LJ. Erythropoietin production in the anephric rat. I. Relationship between nephrectomy, time of hypoxic exposure, and erythropoietin production. Blood. 1972;39:31-38.

4. Fried W. The liver as a source of extrarenal erythropoietin production. Blood. 1972;40:671-677.

5. Wenger RH, Hoogewijs D. Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. Am J Physiol Renal Physiol. 2010;298:F1287-1296.

6. Goldberg MA, Glass GA, Cunningham JM, et al. The regulated expression of erythropoietin by two human hepatoma cell lines. Proc Natl Acad Sci USA. 1987;84:7972-7976.

7. Stolze I, Berchner-Pfannschmidt U, Freitag P, et al. Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells. Blood. 2002;100:2623-2628.

8.Obara N, Suzuki N, Kim K, et al. Repression via the GATA box is essential for tissue-specific erythropoietin gene expression. Blood. 2008;111:5223-5232.

9.Sherwood JB, Shouval D. Continuous production of erythropoietin by an established human renal carcinoma cell line: development of the cell line. Proc Natl Acad Sci USA. 1986;83:165-169.

10. Maxwell PH, Osmond MK, Pugh CW, et al. Identification of the renal erythropoietin-producing cells using transgenic mice. Kidney Int. 1993;44:1149-1162.

11. Plotkin MD, Goligorsky MS. Mesenchymal cells from adult kidney support angiogenesis and differentiate into multiple interstitial cell types including erythropoietin-producing fibroblasts. Am J Physiol Renal Physiol. 2006;291:F902-912.

12. Pan X, Suzuki N, Hirano I, et al. Isolation and characterization of renal erythropoietin-producing cells from genetically produced anemia mice. PLoS One. 2011;6:e25839.

13. Chang YT, Yang CC, Pan SY, et al. DNA methyltransferase inhibition restores erythropoietin production in fibrotic murine kidneys. J Clin Invest. 2016;126:721-731.

14. Bussolati B, Lauritano C, Moggio A, et al. Renal CD133+/CD73+ progenitors produce erythropoietin under hypoxia and prolyl hydroxylase inhibition. J Am Soc Nephrol. 2013;24:1234-1241.

15. Franke K, Kalucka J, Mamlouk S, et al. HIF-1α is a protective factor in conditional PHD2-deficient mice suffering from severe HIF-2α-induced excessive erythropoiesis. Blood. 2013;121:1436-1445.

16. Kurt B, Paliege A, Willam C, et al. Deletion of von Hippel-Lindau protein converts renin-producing cells into erythropoietin-producing cells. J Am Soc Nephrol. 2013;24:433-444.

17. Kurt B, Gerl K, Karger C, et al. Chronic hypoxia-inducible transcription factor-2 activation stably transforms juxtaglomerular renin cells into fibroblast-like cells in vivo. J Am Soc Nephrol. 2015;26:587-596.

18. Gerl K, Miquerol L, Todorov VT, et al. Inducible glomerular erythropoietin production in the adult kidney. Kidney Int. 2015;88:1345-1355.

19. Gerl K, Nolan KA, Karger C, et al. Erythropoietin production by PDGFR-β+ cells. Eur J Physiol. 2016;468:1479-1487.

20. Kobayashi H, Liu Q, Binns TC, et al. Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin. J Clin Invest. 2016;126:1926-1938.

21. Gerl K, Steppan D, Fuchs M, et al. Activation of Hypoxia Signaling in Stromal Progenitors Impairs Kidney Development. Am J Pathol. 2017;187:1496-1511.

22. Bachmann S, Le Hir M, Eckardt KU. Co-localization of erythropoietin mRNA and ecto-5'-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. J Histochem Cytochem. 1993;41:335-341.

23. Matsumoto I, Ohmoto M, Narukawa M, et al. Skn-1a (Pou2f3) specifies taste receptor cell lineage. Nat Neurosci. 2011;14:685-687.

24. Safran M, Kim WY, O'Connell F, et al. Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production. Proc Natl Acad Sci USA. 2006;103:105-110.

25. Guo JK, Shi H, Koraishy F, et al. The Terminator mouse is a diphtheria toxin-receptor knock-in mouse strain for rapid and efficient enrichment of desired cell lineages. Kidney Int. 2013;84:1041-1046.

26. Eckardt KU, Dittmer J, Neumann R, et al. Decline of erythropoietin formation at continuous hypoxia is not due to feedback inhibition. Am J Physiol. 1990;258:F1432-1437.

27. Tan CC, Eckardt KU, Firth JD, et al. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. Am J Physiol. 1992;263:F474-481.

28. Stiehl DP, Bordoli MR, Abreu-Rodríguez I, et al. Non-canonical HIF-2α function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop. Oncogene. 2012;31:2283-2297.

29. Wenger RH, Kurtcuoglu V, Scholz CC, et al. Frequently asked questions in hypoxia research. Hypoxia. 2015;3:35-43.

30. Stiehl DP, Wirthner R, Köditz J, et al. Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem. 2006;281:23482-23491.

31. Wollenick K, Hu J, Kristiansen G, et al. Synthetic transactivation screening reveals ETV4 as broad coactivator of hypoxia-inducible factor signaling. Nucl Acids Res. 2012;40:1928-1943.

32. Lelli A, Nolan KA, Santambrogio S, et al. Induction of long noncoding RNA MALAT1 in hypoxic mice. Hypoxia. 2015;3:45-52.

33. Wenger RH, Stiehl DP, Camenisch G. Integration of oxygen signaling at the consensus HRE. Sci STKE. 2005;2005:re12.

34. Takeda K, Aguila HL, Parikh NS, et al. Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. Blood. 2008;111:3229-3235.

35. Higgins DF, Kimura K, Bernhardt WM, et al. Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. J Clin Invest. 2007;117:3810-3820.

36. Souma T, Nezu M, Nakano D, et al. Erythropoietin synthesis in renal myofibroblasts is restored by activation of hypoxia signaling. J Am Soc Nephrol. 2016;27:428-438.

37. Urrutia AA, Afzal A, Nelson J, et al. Prolyl-4-hydroxylase 2 and 3 coregulate murine erythropoietin in brain pericytes. Blood. 2016;128:2550-2560.

38. Asada N, Takase M, Nakamura J, et al. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. J Clin Invest. 2011;121:3981-3990.

39. Suzuki N, Hirano I, Pan X, et al. Erythropoietin production in neuroepithelial and neural crest cells during primitive erythropoiesis. Nat Commun. 2013;4:2902.

40. Dame C, Kirschner KM, Bartz KV, et al. Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. Blood. 2006;107:4282-4290.

41. Hahne M, Wenger RH, Vestweber D, et al. The heat-stable antigen can alter very late antigen 4-mediated adhesion. J Exp Med. 1994;179:1391-1395.

42. Furness SGB, McNagny K. Beyond mere markers. Immunol Res. 2006;34:13-32.

43. Cretoiu SM, Popescu LM. Telocytes revisited. Biomol Concepts. 2014;5:353-369.

44. Qi G, Lin M, Xu M, et al. Telocytes in the human kidney cortex. J Cell Mol Med. 2012;16:3116-3122.

45. Li L, Lin M, Li L, et al. Renal telocytes contribute to the repair of ischemically injured renal tubules. J Cell Mol Med. 2014;18:1144-1156.

46. Rusu MC, Mogoanta L, Pop F, et al. Molecular phenotypes of the human kidney: Myoid stromal cells/telocytes and myoepithelial cells. Ann Anat. 2018;218:95-104.

47. Bei Y, Zhou Q, Fu S, et al. Cardiac telocytes and fibroblasts in primary culture: different morphologies and immunophenotypes. PLoS One. 2015;10:e0115991.

48. Zheng Y, Zhang M, Qian M, et al. Genetic comparison of mouse lung telocytes with mesenchymal stem cells and fibroblasts. J Cell Mol Med. 2013;17:567-577.

49. Vignais ML, Caicedo A, Brondello JM, et al. Cell connections by tunneling nanotubes: effects of mitochondrial trafficking on target cell metabolism, homeostasis, and response to therapy. Stem Cells Int. 2017;2017:6917941.

50. Koury ST, Koury MJ, Bondurant MC, et al. Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. Blood. 1989;74:645-651.

51. Eckardt KU, Koury ST, Tan CC, et al. Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia. Kidney Int. 1993;43:815-823.

52. Maxwell PH, Ferguson DJ, Nicholls LG, et al. Sites of erythropoietin production. Kidney Int. 1997;51:393-401.

53. Shanks JH, Hill CM, Lappin TR, et al. Localization of erythropoietin gene expression in proximal renal tubular cells detected by digoxigenin-labelled oligonucleotide probes. J Pathol. 1996;179:283-287.

54. Suliman HB, Ali M, Piantadosi CA. Superoxide dismutase-3 promotes full expression of the EPO response to hypoxia. Blood. 2004;104:43-50.

55. Beirao I, Moreira L, Barandela T, et al. Erythropoietin production by distal nephron in normal and familial amyloidotic adult human kidneys. Clin Nephrol. 2010;74:327-335.

56. Nagai T, Yasuoka Y, Izumi Y, et al. Reevaluation of erythropoietin production by the nephron. Biochem Biophys Res Commun. 2014;449:222-228.

57. Loya F, Yang Y, Lin H, et al. Transgenic mice carrying the erythropoietin gene promoter linked to lacZ express the reporter in proximal convoluted tubule cells after hypoxia. Blood. 1994;84:1831-1836.

58. Haidar MA, Loya F, Yang Y, et al. Electron microscopic localization of lacZ expression in the proximal convoluted tubular cells of the kidney in transgenic mice carrying chimeric erythropoietin/lacZ gene constructs. J Struct Biol.

59. Farsijani NM, Liu Q, Kobayashi H, et al. Renal epithelium regulates erythropoiesis via HIF-dependent suppression of erythropoietin. J Clin Invest. 2016;126:1425-1437.

60. Semenza GL, Koury ST, Nejfelt MK, et al. Cell-type-specific and hypoxia- inducible expression of the human erythropoietin gene in transgenic mice. Proc Natl Acad Sci USA. 1991;88:8725-8729.

61. Nolan KA, Wenger RH. Source and microenvironmental regulation of erythropoietin in the kidney. Curr Opin Nephrol Hypertens. 2018;27:277-282.

62. Yamazaki S, Souma T, Hirano I, et al. A mouse model of adult-onset anaemia due to erythropoietin deficiency. Nat Commun. 2013;4:1950.

63. Olmos G, Muñoz-Félix JM, Mora I, et al. Impaired erythropoietin synthesis in chronic kidney disease is caused by alterations in extracellular matrix composition. J Cell Mol Med. 2018;22:302-314.

64. Souma T, Suzuki N, Yamamoto M. Renal erythropoietin-producing cells in health and disease. Front Physiol. 2015;6:167.

65. Suzuki N, Yamamoto M. Roles of renal erythropoietin-producing (REP) cells in the maintenance of systemic oxygen homeostasis. Eur J Physiol. 2016;468:3-12.

66. Haase VH. Therapeutic targeting of the HIF oxygen-sensing pathway: Lessons learned from clinical studies. Exp Cell Res. 2017.

67. Humphreys BD, Lin SL, Kobayashi A, et al. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol. 2010;176:85-97.

68. Marti HH, Wenger RH, Rivas LA, et al. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci. 1996;8:666-676.

69. Bernaudin M, Bellail A, Marti HH, et al. Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. Glia. 2000;30:271-278.

70. Johansson T, Broll I, Frenz T, et al. Building a zoo of mice for genetic analyses: a comprehensive protocol for the rapid generation of BAC transgenic mice. Genesis. 2010;48:264-280.

71. Nytko KJ, Maeda N, Schläfli P, et al. Vitamin C is dispensable for oxygen sensing in vivo. Blood. 2011;117:5485-5493.

72. Schörg A, Santambrogio S, Platt JL, et al. Destruction of a distal hypoxia HIF-response element abolishes trans-activation of the PAG1 gene mediate by independent chromatin looping. Nucl Acids Res. 2015;43:5810-5823.

5. Single cell variability of hypoxia-inducible gene expression

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Introduction

Hypoxia is a condition occurring when the oxygen demand exceeds the oxygen supply and represents a harmful threat for the physiological functions of our body. As multicellular organisms need a constant supply of oxygen for their survival, they evolved specific mechanisms based on systemic and cellular responses by which to adapt to hypoxia [1]. The master regulator of this adaptation is the transcription factor hypoxia-inducible factor (HIF), discovered in hepatoma cells by its binding to a hypoxia response element (HRE) located downstream of the erythropoietin gene (*EPO*) [2] [3]. HIF controls the activation of hundreds of genes involved in several cellular processes that need to adapt to oxygen deprivation such as glucose metabolism, angiogenesis, erythropoiesis, iron uptake and cell proliferation [4]. Within the HIF pathway, the three prolyl-4-hydroxylase domain (PHD1/2/3) enzymes, the asparagine hydroxylase factor inhibiting HIF (FIH) and the ubiquitin E3 ligase von Hippel Lindau (VHL) protein recognizing hydroxylated HIF-α subunits, have been discovered to play a crucial role in regulating HIF-α protein stability, hence, modulating the HIF pathway activation by sensing oxygen levels [5] [6] [7]. The two main HIF isoforms, HIF-1 and HIF-2 recognise and bind the same DNA consensus sequence, the so-called HIF binding site (HBS) within the HRE but their target genes are only partially overlapping [7] [8]. Interestingly, HIF-1α and HIF-2α recruit diverse transcriptional co-activators in a cell-type specific manner and show different kinetics of protein stabilization, indicating that the duration of the hypoxic exposure needs to be considered in target gene expression studies. Despite many cell lines co-express both HIF-1α and HIF-2α, it has been observed by knock-down experiments that suppression of either isoform increases the abundance of the other isoform [9]. This mechanism of mutual inhibition, probably evolved to gain more flexibility in the hypoxic response, is still poorly understood as in several batch systems both HIF-α isoforms are still co-expressed; thus, it needs to be investigated by a single cell-based approach.

Erythropoietin (Epo) is the main hormone maintaining the RBC homeostasis and its regulation occurs mostly on the mRNA level in a HIF-2 dependent manner. While the fetal liver is the organ synthetizing Epo during development, the kidney is the main organ

producing Epo during adulthood [10] [8]. Ectopic Epo expression can also be found in cells belonging to brain, uterus and testis but they do not substantially contribute to circulating Epo [11]. In the kidney, Epo is synthetized by juxtamedullary cortical interstitial cells, termed renal Epo-producing (REP) cells, which display features of fibroblasts, pericytes and neurons [8] [11] [12]. The availability of hepatoma and neuroblastoma cell lines, capable of producing Epo in a hypoxia inducible manner, allowed the study of oxygen-regulated Epo transcriptional regulation in both liver- and brain-derived *in vitro* models [13]. However, these insights cannot be extended to renal Epo transcriptional regulations as no reliable cell culture model for REP cells was available until recently. In fact, previous reporter renal cell lines did not display regulated Epo expression and kidney-derived cells from knock-in mice containing a SV40 large T antigen within the *Epo* locus did not permanently express Epo [14]. Therefore, to study endogenous renal Epo transcriptional regulation, we generate a novel transgenic mouse model to conditionally tag for active *Epo* locus and isolate fresh renal Epo producing cells as new reliable renal cell culture system. Three clonal Epo-producing fibroblastoid atypical interstitial kidney (FAIK) cell lines deriving from “on” tagged mouse primary REPCs were generated in our lab that showed HIF-2α-dependent Epo kinetics in a similar way to the *in vivo* kinetics [15]. While immunoistochemical approaches and “classical’’ *in situ* hybridization methods displayed Epo expression not only in interstitial peritubular renal cells but also in other cell types of the kidney, the recent development of single mRNA molecule detection by chromogenic (HRP-DAB) and fluorescent (FISH) detection methods (RNAscope) [16], allowed us and other groups to finally demonstrate that Epo mRNA is expressed only in a subpopulation of juxtamedullary cortical interstitial cells [15] [17]. Because both renal Epo mRNA and circulating Epo protein transiently increase upon hypoxia by hundred-folds [21], it is generally thought that, depending on the presence or absence of Epo mRNA, REPCs can be either in an “on” or “off” state, indicating Epo-producing or non Epo-producing state, respectively [10] [11]. Thus, REP cells are characterized by high cell-to-cell variability, a feature that under hypoxia has not been yet studied. In normoxic conditions, cell-to-cell variability has been demonstrated to be determined by on-off kinetics and “bursty” transcription [19] [20]. This raises the question about the nature of Epo transcription in hepatoma, neuroblastoma and REP cells. It would be of great interest to examine whether the induction of Epo, (or more generally, the induction of a given HIF target gene) in a single cell is completely random (stochastic) or whether currently unknown factors influence the cellular adaptation to the hypoxic environment (deterministic) and if its expression is bursty or continuous. Additionally, it remains still unclear whether the loss of Epo production is caused by a definitive differentiation toward non Epo-producing cells or a transient switch to the “off” state, which might be reverted to “on”, reflecting the physiological switch of Epo expression in response to the hypoxic stimulus. Of note, despite severe hypoxic conditions (8% O_2 for 3

hours), only a small fraction of interstitial cells located at the renal cortex and outer medulla initiated Epo transcription, implying that Epo transcription is bursty rather than continuous [15]. Taken together, such open questions suggest that Epo expression needs to be studied on a single cell level as so far, *in vitro* Epo transcription has been analysed only in batch-experiments, neglecting the transient “on-off behaviour”.

Therefore, in this work, we aimed to understand the impact of oxygen levels and time of hypoxic exposure (extrinsic factors) as well as mRNA distribution (intrinsic factor) on the cell-to-cell variability of the “hypoxic transcript pattern”, defined as the cellular distribution of single nascent and mature mRNA, in Epo-producing hepatoma, neuroblastoma and non-transformed renal mouse (FAIK) cell lines by chromogenic *in situ* hybridization of single mRNA molecule (RNAscope).

Material and Methods

Cell culture

Human Hep3B and HepG2 hepatocellular carcinoma (American Type Culture Collection, LGC Standards, Wesel, Germany) and Kelly neuroblastoma (kindly provided by J. Fandrey, Essen, Germany) cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium, respectively (Sigma Aldrich, Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA USA). Cells were exposed to hypoxic conditions using a workstation as described previously [21].

In situ hybridization

Epo mRNA was detected by *in situ* hybridization according to the manufacturer’s instructions (RNAscope; Advanced Cell Diagnostics, Hayward, CA, USA) as reported previously [303]. Briefly, the human Epo (Cat No. 414201) and the mouse Epo (Cat No. 315501) consist of 12 double Z probe pairs and target the region between 252 and 1334 as well as 39 and 685 of human and mouse Epo, respectively. Human PPIB (Cat No. 313901) and mouse Ppib (Cat No. 31391) as well as DapB (Cat No. 310043) were used as positive and negative control probes, respectively. Cells were fixed with 4% PFA on a 8-chamber glass dishes (Lab-Tek; Thermo Fisher Scientific) and prepared according to the manufacturer’s recommendations. The probes were hybridized for 2 hours at 40°C in a HybEZ oven (Advanced Cell Diagnostics, Hayward, CA, USA), followed by a series of signal amplification and washing steps. For horseradish peroxidase (HRP) labelled hybridization probes, signals were detected with 3,3'-diaminobenzidine (DAB) using the 2.5 HD reagent kit BROWN (Advanced Cell Diagnostics). FastRed turnover by alkaline phosphatase was detected using the 2.5 HD

reagent kit RED (Advanced Cell Diagnostics) [16]. Cells were counterstained with 50% Gill's hematoxylin, dehydrated by baking at 60°C for 15 minutes and mounted with the xylene based mounting medium Eukitt (Sigma Aldrich, Saint Louis, MO, USA).

Microscopy and data analysis

Cells were imaged at bright field with a Nikon Eclipse TE300 inverted microscope or a slide scanner (Axio Scan.Z1; Zeiss) (Center for Microscopy and Image Analysis, University of Zurich). Quantitative image analysis was performed by selecting randomly ~3 visual fields per each slide including ~ 500 cells. The number of cells and mRNA molecules (brown or fluorescent dots) per cell were counted by using the open-source cell image analysis software CellProfiler [22]. GraphPad Prism software was used for the preparation of the displayed graphs.

Results

To characterize the hypoxic Epo transcript pattern on a single cell level, we performed chromogenic Epo mRNA *in situ* hybridization (ISH) (RNAscope) on the well-established human Epo-producing Hep3B and HepG2 hepatoma and Kelly neuroblastoma as well as on our recently generated immortalized but non-transformed renal mouse FAIK3-5 cell line. Cultured cells were seeded on 8-chamber glass dishes and incubated in normoxia (21% O_2) or hypoxia (0.2% O_2) for 24 hours or 8 hours (only HepG2). To verify the functionality of the ISH method in our conditions, positive and negative controls, the constitutively expressed low-copy housekeeping gene PPIB (peptidylprolyl isomerase B) and the bacterial gene dapB (4-hydroxy-tetrahydrodipicolinate reductase), respectively, were included. To quantify the number of cells and the number of mRNA targets per cell, computer-assisted and cell line specific-profiles were designed by using the Cell Profiler software.

As shown in Figure 1E, the housekeeping gene PPIB was homogenously and highly expressed in HepG2, whereas the bacteria gene dapB was absent, indicating that no bacteria contamination was present during cell culture. In HepG2 cells, Epo mRNA was successfully induced under hypoxia at both 8 and 24 hour time points as highlighted by the numerous dots in Figure 1 A-B. Surprisingly, HepG2 cells did not show a homogenous expression of Epo mRNA but rather a cell-to-cell variability suggested by the fact that, while some cells display many Epo mRNA molecules (brown dots), some other show less dots per cell. As shown by the histograms of Figure 2A, about 40% of hypoxic HepG2 cells display one Epo mRNA molecule, whereas ~20% of the HepG2 express 21 dots per cell at both at 8 and 24 hours. Only less than 10% of cells resulted to express more than 100 dots per cells. To investigate the hypoxic transcript pattern of other HIF target genes, we focused on the

HIF-2α target gene PHD3 (prolyl-4hydroxylase domain 3) as involved, together with PHD2, in forming a negative feedback loop that has been reported to reverse the hypoxic response but that it is poorly characterized at the molecular level. For PHD3, we observed a similar hypoxic transcript pattern as found for Epo (Figure 1C-D) with uneven distribution of PHD3 mRNA molecules among hypoxic HepG2 cells (Figure 2B). The negative control gene dapB was histogram-quantified to verify the reliability of the cell type-specific software profile that was assessed by manual comparison between imaged and quantified dot (Figure 2C). Successively, we aimed to visualize and quantify Epo mRNA single molecules in Hep3B cells. In contrast to HepG2, hypoxic Hep3B cells displayed generally lower gene levels as seen by reduced amounts of dots associated with Epo, PHD3 and PPIB mRNA molecules. However, in line with HepG2, the “cell-to-cell behaviour” was observed in Hep3B as well, indicating that both hepatoma cell lines express hypoxic genes in a cell-to-cell variable manner compared to more homogenous expression of the PPIB housekeeping gene.

Unexpectedly, Kelly neuroblastoma cells, which express 10-fold and 100-fold more Epo transcripts compared to Hep3B and HepG2, respectively, showed a more profound non-homogenous Epo mRNA expression pattern. While hepatoma cells displayed a dotted Epo and PHD3 mRNA pattern that was unambiguously quantified, the signal detected in Kelly was more intense and presented an undefined non-dotted shape (Figure 4A-B) that was not possible to quantify by computer-assisted method. Consistently, also the PPIB gene was highly expressed with non-dotted signal feature (Figure 4C).

We, next, validated the ISH method in FAIK cells, which were recently generated in our lab and that showed HIF-2α-dependent Epo kinetics. As our first attempt in these non-transformed cells, the detection of mEpo and mHIF-2α was, unfortunately, inefficient due to oxygen-unregulated and weak signal (data not shown). However, we could successfully detect mHIF-1α, mPHD2, mPHD3 (Figure 5A) and mVEGF (Figure 5B). Especially, we observed hypoxic regulation of mPHD2 and mPHD3 after 6 hours (peak of Epo induction in FAIK cells), whereas mHIF-1α expression resulted to be stable between both hypoxic and normoxic conditions, consistently with a regulation of HIF-1α at protein level. Similarly to hepatoma and neuroblastoma, FAIK cells also showed high cell-to-cell variability as highlighted by the irregular distribution of dots per cell (Figure 5A-B). Like REPCs, FAIK cells display long projections, probably required to form cell-cell contacts in the interstitial space between blood vessel and renal tubules. Intriguingly, we could observed mHIF-1α mRNA molecules within these projections in either hypoxic or normoxic conditions (Figure 5).

Discussion

In this work, we established a recently developed ISH chromogenic method, ensuring the detection of single mRNA molecules that are imaged as single dots [16] in hepatoma, neuroblastoma and non-transformed FAIK cell lines to investigate the hypoxic transcript pattern of Epo and other HIF target genes on a single cell level. By comparing the transcript pattern of hypoxia-inducible genes to the constitutively expressed housekeeping gene PPIB, we observed, for the first time, that differences between these two different classes of genes exist, with a strong cell-to-cell variability of Epo and the HIF-2α target gene PHD3 in HepG2, Hep3B, Kelly and FAIK cells. Particularly, these cell lines showed a heterogeneous distribution of mRNA molecules with cells having a complete absence of target mRNA molecules, cells having little amount of mRNAs or cells with massive number of transcripts, implying that few cells produce most of the mRNA molecules. While for HepG2 and Hep3B we could faithfully quantify the distribution of single mRNA molecules per number of cell by computer-assisted approach, we were, unfortunately, unsuccessful with Kelly and FAIK cells. Specifically, we experienced difficulties in designing a personalized profile for Kelly cells as their strong chromogenic signal lost the dotted pattern during its accumulation and expansion among the cells. Additionally, we had problems in optimizing the FAIK cells profile as their typical long processes were not counted by Cell profiler when drawing the cell border. Therefore, we are planning to repeat the *in situ* hybridization in both Kelly and FAIK cells by using a fluorescence-based ISH with higher resolution that allows the detection of simultaneous mRNA targets as well as the design of a new profile to precisely quantify the fluorescent dots representing the mRNA transcripts. Surprisingly, in FAIK cells we could detect mRNA transcripts within their thin processes, indicating that, eventually, they might have a role in transferring mRNA molecules to other cells by interacting through cell-cell contacts. However, we have no evidence to prove this hypothesis so far and further investigations are currently ongoing in our group. Nevertheless, it is without precedent that single mRNA molecules are observed within the processes of renal interstitial cells. Taken together, our first attempt in performing a chromogenic ISH *in vitro,* provided evidence for a not yet reported strong cell-to-cell variability of the hypoxic transcript pattern of HIF target genes but not housekeeping genes.

Our future plans include hypoxia titration in order to test oxygen sensitivity of hepatoma, neuroblastoma and FAIK cells to test which is the best oxygen concentration for Epo mRNA induction and how the Epo transcription pattern is affected by graded hypoxia. In addition to Epo, we would like to analyse also HIF-α isoform specific target genes such as CAIX and mVEGF (HIF-1α target gene) and PHD2 and PHD3 (HIF-2α target genes). It would be also interesting to perform hypoxia kinetics studies in Hep3B, HepG2, Kelly and FAIK cells to

verify whether the pattern of cell-to-cell variability changes with ongoing hypoxia. Since we found that mVEGF mRNA was strongly localized in the nucleus of hypoxic FAIK cells (Figure 5B), we would like to investigate this observation by elucidating the nuclear hypoxic transcription initiation pattern. Especially, we would like to understand whether the nuclear hypoxic transcription initiation pattern determines the occurring hypoxic transcription pattern or whether post-transcriptional events influence the cell-to-cell variability of the HIF target genes. We would approach this question by designing intronic ISH probes that should be detected only in the nucleus. Then, it would be intriguing to compare the nuclear hypoxic transcription initiation pattern with the general hypoxic transcript pattern for a given HIF target gene in each cell line. Importantly, we generated in our group Hep3B, HepG2 and Kelly cells with endogenous mutations within the 5' or 3' hypoxia response element (HRE) and could demonstrate that while in hepatoma the 3' HRE is the only element required for hypoxic Epo induction, in Kelly cells both the 5' and the 3' HREs enhance hypoxic Epo transcription in cooperation with the newly discovered promoter HREs (see Manuscript I). The availability of such mutant clones gives us the chance to study the cell-to-cell variability of the hypoxic Epo transcript pattern in a context of mutual functionality of the HIF-dependent (3' HRE and promoter HREs) and the HIF-independent (5' HRE) enhancers to verify whether Epo HREs contribute to its hypoxic transcription pattern. Finally, because HIF-1α and HIF-2α knock-down batch experiments suggest a mutual inhibition behaviour showing up-regulation of one isoform when the other is down-regulated [9], we are interested in testing whether at the single cell level both isoforms can be expressed equally. For this experiment we would opt for a fluorescent ISH method in order to simultaneously image both isoforms in Hep3B, HepG2, Kelly wt cells or stable HIF-1α and HIF-2α knock-down cells. The latter condition will be useful to validate whether down-regulation of the respective HIF-α isoforms influence the HIF-1α and HIF-2α transcription patterns.

In conclusion, this work was a first attempt to apply the chromogenic ISH method to cultured cells in order to study the hypoxic transcription pattern of Epo and other relevant HIF target genes. We could successfully detect Epo transcripts in hepatoma and neuroblastoma as well as quantify the distribution of the mRNA molecules per cell in both Hep3B and HeG2. Finally, by this new ISH method, we provided initial *in vitro* evidence for inherent cell-to-cell variability of the hypoxic transcript pattern in hepatoma, neuroblastoma and FAIK cells.

References

1. Taylor, C.T. and J.C. McElwain, *Ancient atmospheres and the evolution of oxygen sensing via the hypoxia-inducible factor in metazoans.* Physiology (Bethesda), 2010. **25**(5): p. 272-9.

2. G L Wang, B.H.J., E A Rue, and G L Semenza, *Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension.* Proc Natl Acad Sci U S A. , 1995.

3. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5680-4.

4. Wenger, R.H., *Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression.* FASEB J, 2002. **16**(10): p. 1151-62.

5. Schofield, C.J. and P.J. Ratcliffe, *Oxygen sensing by HIF hydroxylases.* Nat Rev Mol Cell Biol, 2004. **5**(5): p. 343-54.

6. Kaelin, W.G., Jr. and P.J. Ratcliffe, *Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway.* Mol Cell, 2008. **30**(4): p. 393-402.

7. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE.* Sci STKE, 2005. **2005**(306): p. re12.

8. Wenger, R.H. and D. Hoogewijs, *Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells.* Am J Physiol Renal Physiol, 2010. **298**(6): p. F1287-96.

9. Stiehl DP, B.M., Abreu-Rodríguez I, Wollenick K, Schraml P, Gradin K, Poellinger L, Kristiansen G, Wenger RH. , *Non-canonical HIF-2alpha function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop.* Oncogene, 2012.

10. Jelkmann, W., *Regulation of Erythropoietin production.* J Physiolo, 2011.

11. Souma, T., N. Suzuki, and M. Yamamoto, *Renal erythropoietin-producing cells in health and disease.* Front Physiol, 2015. **6**: p. 167.

12. Suzuki, N. and M. Yamamoto, *Roles of renal erythropoietin-producing (REP) cells in the maintenance of systemic oxygen homeostasis.* Pflugers Arch, 2016. **468**(1): p. 3-12.

13. Goldberg, M.A., et al., *The regulated expression of erythropoietin by two human hepatoma cell lines.* Proc Natl Acad Sci U S A, 1987. **84**(22): p. 7972-6.

14. Maxwell PH, O.M., Pugh CW, Heryet A, Nicholls LG, Tan CC, Doe BG, Ferguson DJ, Johnson MH, Ratcliffe PJ., *Identification of the renal erythropoietin-producing cells using transgenic mice.* Kidney Int. , 1993.

15. Imeri, F., et al., *Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation, and a telocyte phenotype.* Kidney Int, 2019. **95**(2): p. 375-387.

16. Wang, F., et al., *RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues.* J Mol Diagn, 2012. **14**(1): p. 22-9.

17. Kobayashi, H., et al., *Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin.* J Clin Invest, 2016. **126**(5): p. 1926-38.

18. Wenger, R.H. and A. Kurtz, *Erythropoietin.* Compr Physiol, 2011. **1**(4): p. 1759-94.

19. Bahar Halpern, K., et al., *Bursty gene expression in the intact mammalian liver.* Mol Cell, 2015. **58**(1): p. 147-56.

20. Skinner, S.O., et al., *Single-cell analysis of transcription kinetics across the cell cycle.* Elife, 2016. **5**: p. e12175.

21. Schorg, A., et al., *Destruction of a distal hypoxia response element abolishes trans-activation of the PAG1 gene mediated by HIF-independent chromatin looping.* Nucleic Acids Res, 2015. **43**(12): p. 5810-23.

22. Carpenter, A.E., et al., *CellProfiler: image analysis software for identifying and quantifying cell phenotypes.* Genome Biol, 2006. **7**(10): p. R100.

Figure 1

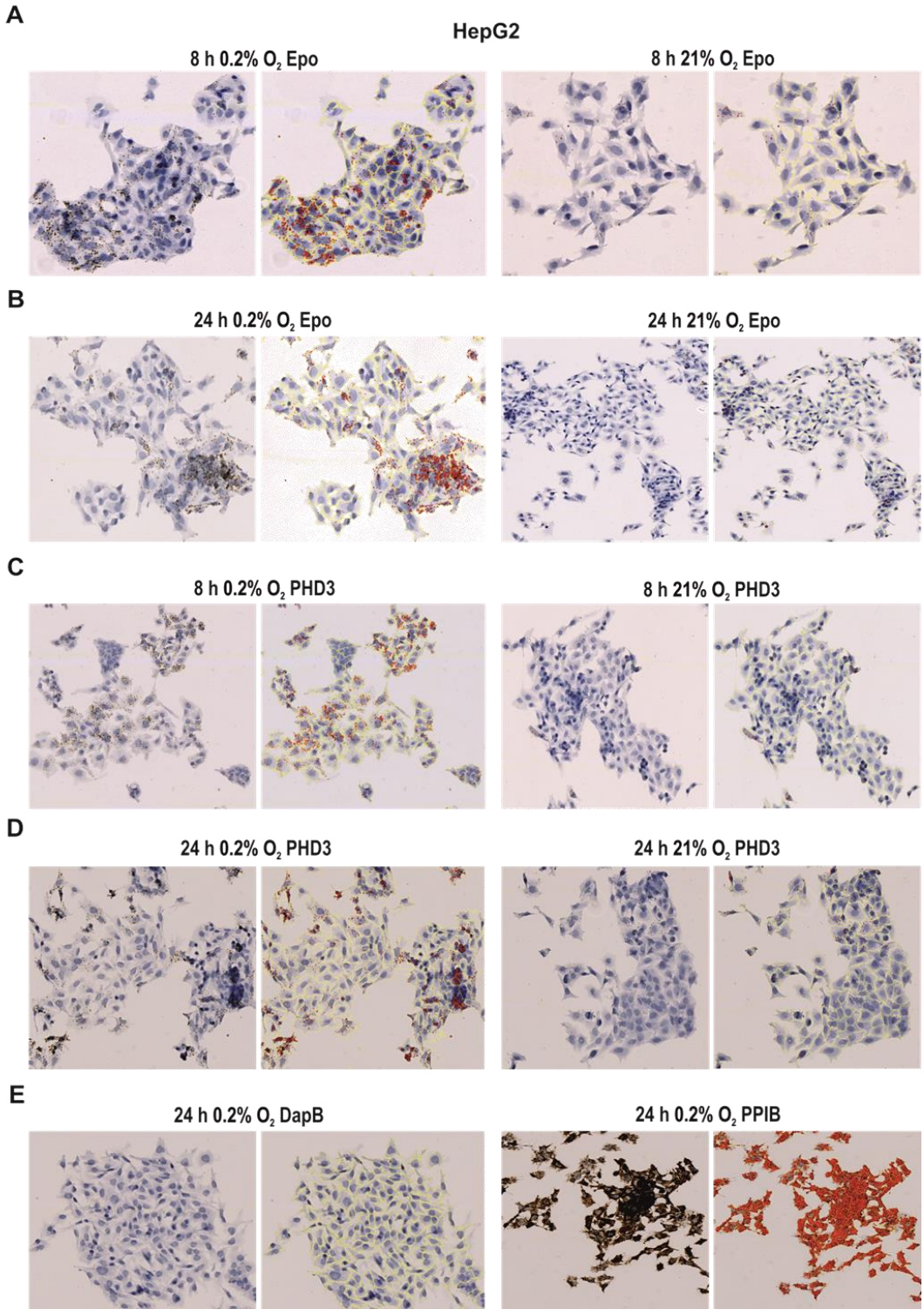

Figure 1. Single cell analysis of the hypoxic transcript pattern in human HepG2 hepatoma cells. RNAscope ISH technology (histochemical HRP-DAB stain with hematoxylin counterstain) was applied to *in vitro* cultured cells exposed to normoxic or hypoxic conditions for 8 or 24 hours as indicated. (A,B) Example ISH for human Epo and PHD3 (C,D) in HepG2 cells are shown. (E) The bacterial negative control gene DapB and the housekeeping positive control gene PPIB in normoxic condition are shown. Hypoxic levels (not shown) of DapB and PPIB were indistinguishable. The image processed by Cell profiler is shown next to the bright field pictures. Yellow lines delineate cell borders and the red circles indicate single mRNA transcripts detected by the software.

Figure 2

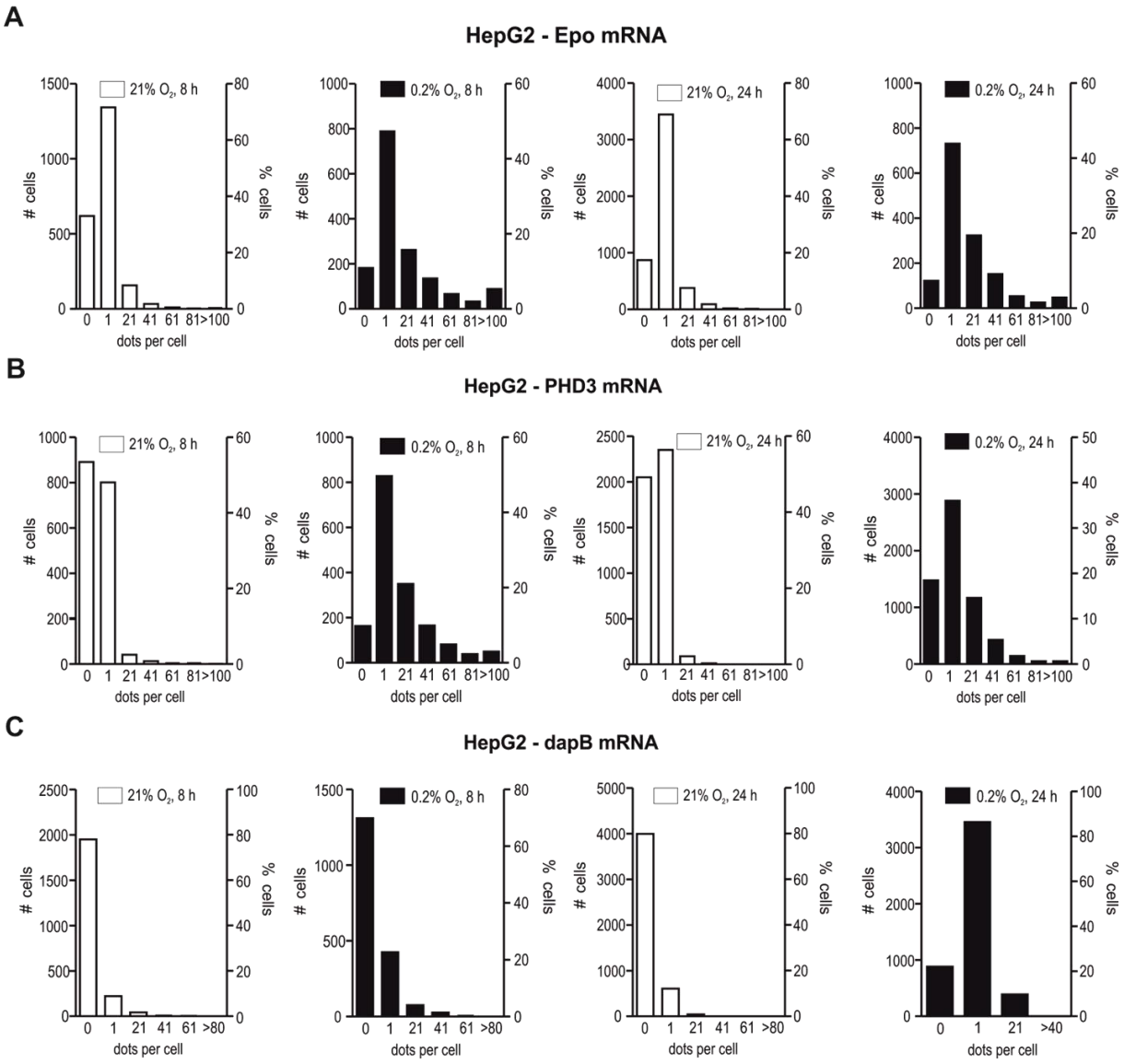

Figure 2. Quantification of the mRNA molecules per number and percentage of HepG2 cells. (A) Histogram analysis of single-cell mRNA distribution for Epo (A), PHD3 (B) and the negative control gene DapB (C) used to assess the functionality of the quantification method. (A,B,C) The y-axis provides absolute number of cells (#), while the right axis provides the percentage cell number (%).

Figure 3

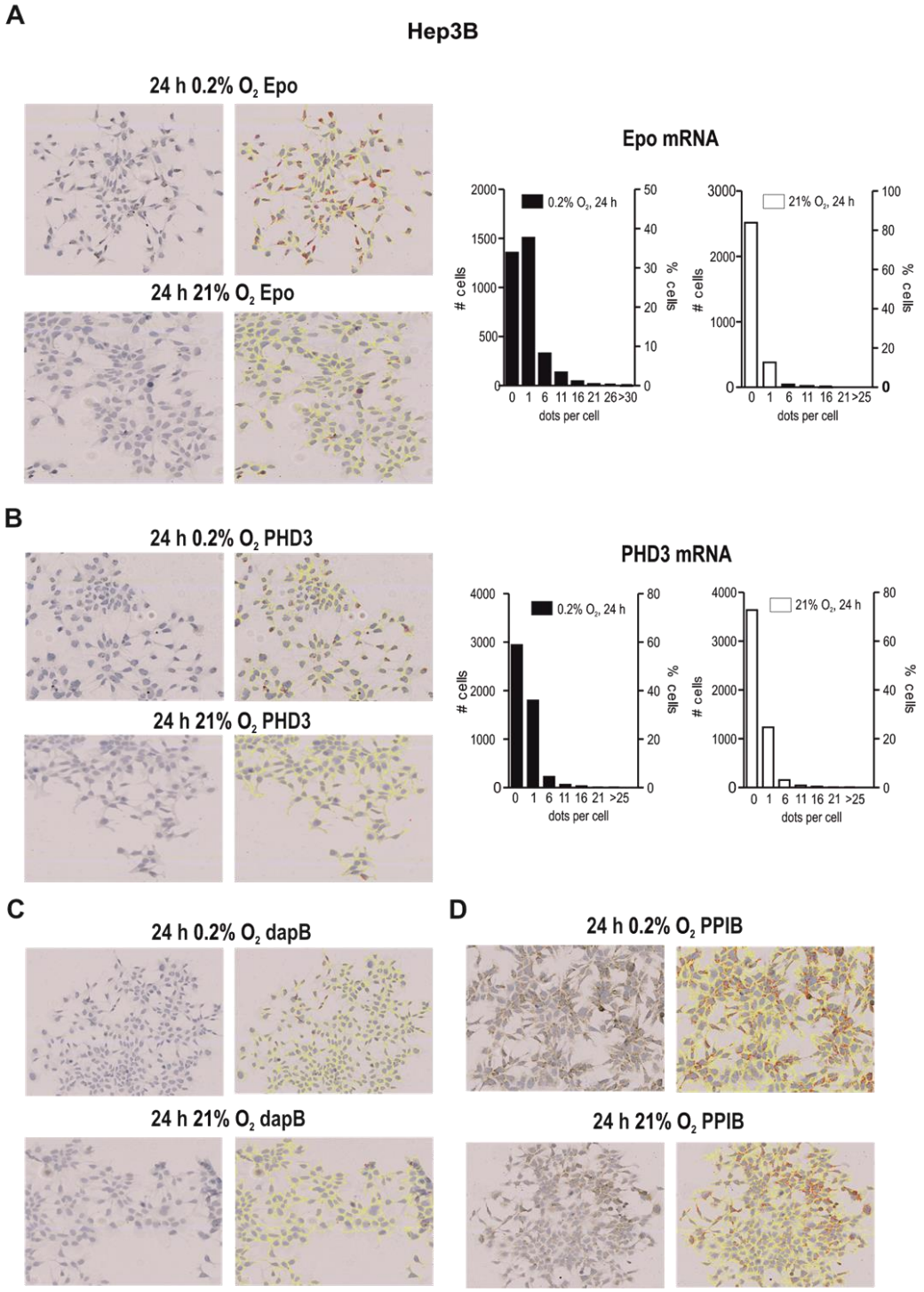

Figure 3. Single cell analysis of the hypoxic transcript pattern in human Hep3B hepatoma cells. RNAscope ISH technology (histochemical HRP-DAB stain with hematoxylin counterstain) was applied to *in vitro* cultured cells exposed to normoxic or hypoxic conditions for 24 hours as indicated. Example ISH for human Epo (A) and PHD3 (B) in Hep3B cells are shown. Histogram analysis of single-cell mRNA distribution for Epo (A) and PHD3 (B) are displayed. The y-axis provides absolute number of cells (#), while the right axis provides the percentage cell number (%). The bacterial negative control gene DapB (C) and the housekeeping positive control gene PPIB (D) in normoxic and hypoxic conditions are shown. The image processed by Cell profiler is shown next to the bright field pictures. Yellow lines delineate cell borders and the red circles indicate single mRNA transcripts detected by the software.

Figure 4

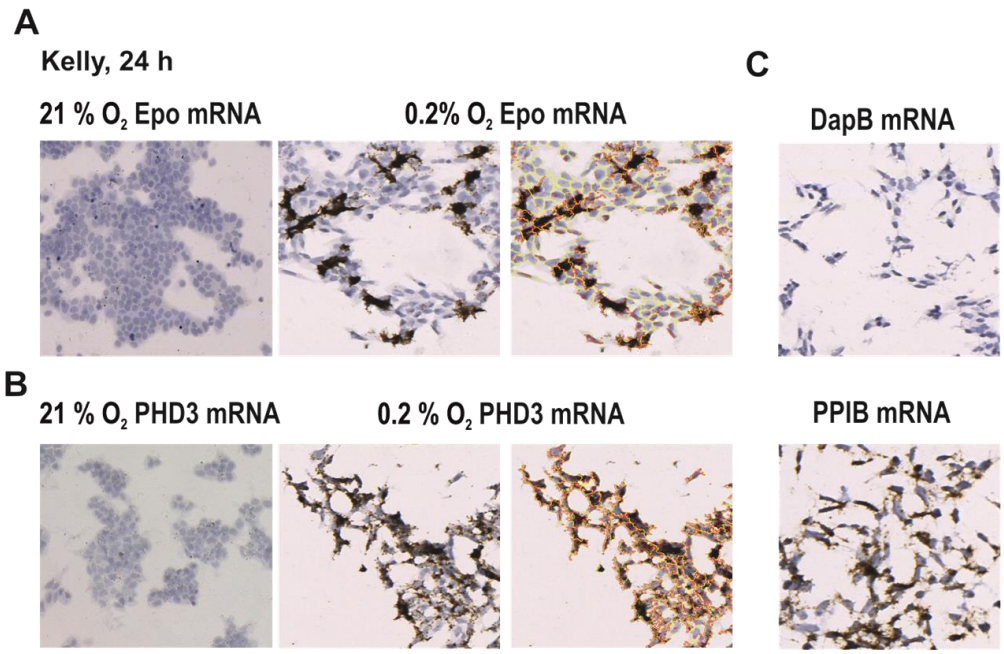

Figure 4. Single cell analysis of the hypoxic transcript pattern in human Kelly neuroblastoma cells. RNAscope ISH technology (histochemical HRP-DAB stain with hematoxylin counterstain) was applied to *in vitro* cultured cells exposed to normoxic or hypoxic conditions for 24 hours as indicated. Example ISH for human Epo (A) and PHD3 (B) in Kelly cells are shown. (C) The bacterial negative control gene DapB and the housekeeping positive control gene PPIB in hypoxic condition are shown. Normoxic levels (not shown) of DapB and PPIB were indistinguishable.

Figure 5

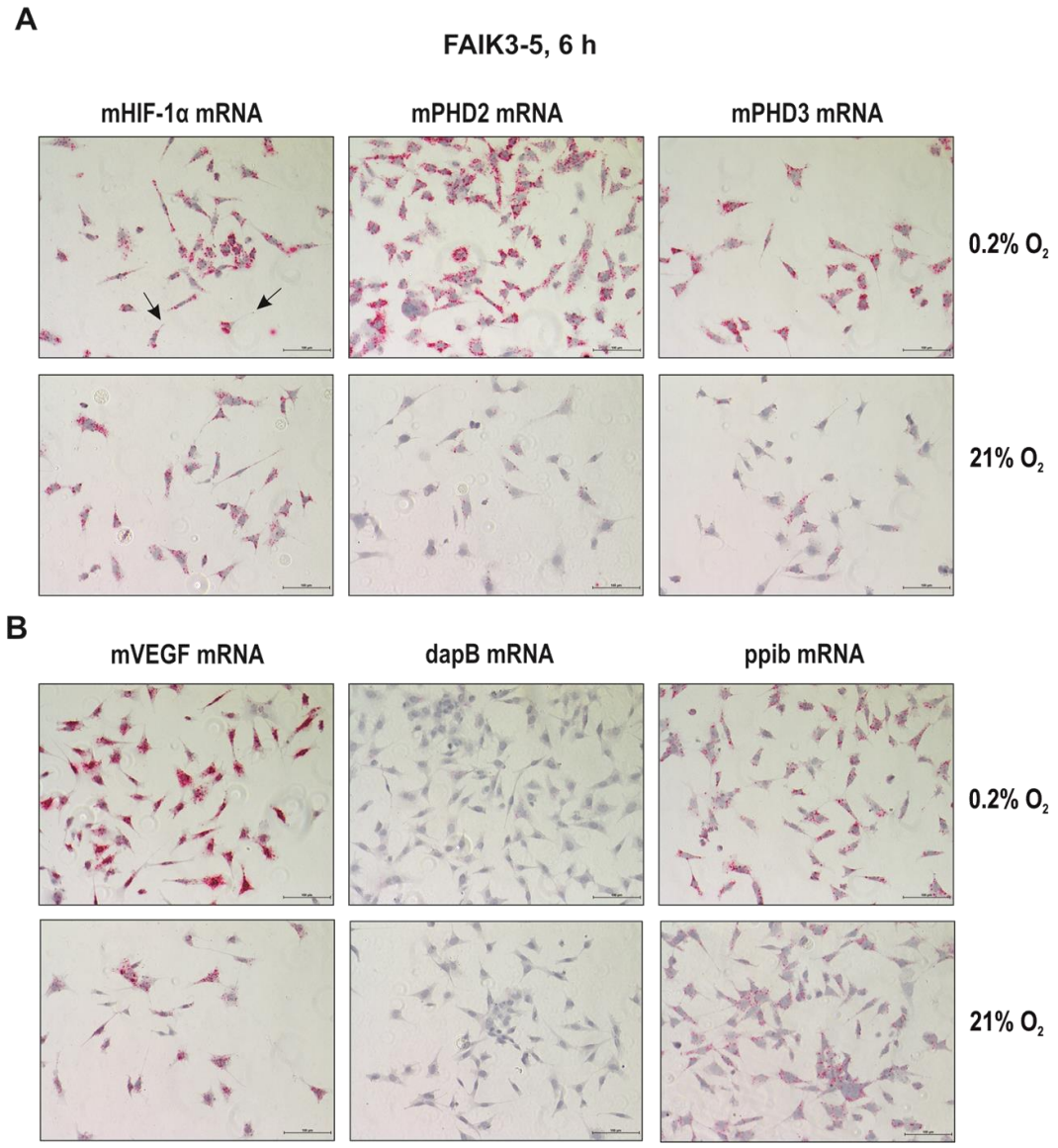

Figure 5. Single cell analysis of constitutive (mHIF-1α, ppib) and hypoxically induced (mPHD2, mPHD3, mVEGF) in mouse FAIK3-5 cells. RNAscope ISH technology (histochemical AP-FastRed stain with hematoxylin counterstain) was applied to *in vitro* cultured cells exposed for 6 hours in hypoxia as indicated. (A) Arrows indicate HIF-1α mRNA in processes. Note that mVEGF but not the other mRNA target genes show a pronounced nuclear staining pattern.

6. Conclusions and future perspectives

Oxygen is essential for the survival of all aerobic organisms. In mammals, oxygen sensing through the prolyl-4-hydroxylase domain (PHD1/2/3) enzymes is fundamental to drive the activation of the HIF pathway that controls the adaptation to hypoxia, a condition in which oxygen demand exceeds the oxygen supply [1]. The master regulator of this adaptation, the transcription factor hypoxia-inducible factor (HIF) has been discovered in hepatoma cells by its binding to the 3' hypoxia response element (HRE) located downstream of the erythropoietin (*EPO*) gene [2]. Epo is the main regulator of red blood cell homeostasis. The main Epo producing organs are the fetal liver and the adult kidney but ectopic Epo expression is found also in other organs including the brain as Epo has either erythropoietic or non-erythropoietic functions [3]. Importantly, *EPO* is a unique example of a gene regulated in a hypoxia-inducible and tissue-specific manner. In fact, distinct regulatory tissue-specific DNA elements enhance EPO transcription in liver and kidney, whereas Epo regulation in the brain is completely unexplored. Previous *in vivo* studies demonstrated that while the 3' HRE is necessary and sufficient for liver-specific *EPO* induction after embryonic day 14.5 [4], the essential regulatory element for Epo regulation in the kidney resides between -14 and -6 kb upstream of the transcription start site (TSS) of the *EPO* gene [5] [6]. However, the precise molecular mechanism underlying the transcriptional regulation of erythropoietin in the kidney remains undefined due to the lack of an appropriate renal cell culture system capable of hypoxia-inducible Epo expression [7]. Given the above observations, the aim of the thesis was to investigate the DNA regulatory elements modulating the transcriptional regulation of erythropoietin in hypoxia in well-established liver- and brain-derived *in vitro* cell culture models.

In particular, we were able to dissect the contribution of the distal 5' and 3' HREs as well as the promoter to endogenous *EPO* gene expression in hepatoma and neuroblastoma cell lines by employing the cluster regularly interspace short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) technique (CRISPR/Cas) to mutate the novel 5' and the well-established 3' HRE generating single (Hep3B, HepG2, Kelly) and double mutant clones (Kelly).

Role of distal 5' and 3' HREs and promoter in regulating hypoxia-inducible Epo in hepatoma and neuroblastoma cell lines

We recently discovered a strongly conserved distal 5' HRE within a DNase I hypersensitive site located at -9.2 kb upstream of the EPO TSS, containing both the 5'-ACGTG-3' core HIF DNA binding site as well as the ancillary 5'-CACA-3' element. We found that this 5' HRE is

functional in hypoxia as it drives hypoxia-inducible exogenous reporter gene expression in both Epo expressing and non-expressing cell lines. Therefore, we suggested that the novel 5' HRE could be the putative oxygen-regulated DNA element modulating renal Epo expression [8]. Due to the lack of a renal Epo-producing cell culture model and considering the neuronal features that REPCs possess, we analysed the relative contribution of the 5' and 3' HREs in Kelly cells, a human neuroblastoma cell line capable of producing Epo in a hypoxia-inducible manner that might recapitulate Epo transcriptional regulation in the brain [9]. While we confirmed that the 3' HRE is the only required element regulating Epo induction in hepatic cells, in neuronal cells, both the 5' and 3' HREs appear to be important to regulate hypoxic Epo induction. In contrast to Hep3B, where the Epo promoter is only slightly bound by HIF and no hypoxic induction could be detected in reporter gene assays, in Kelly cells, surprisingly, the Epo promoter is the DNA element that is most strongly bound by HIF, in line with a weak hypoxic induction observed in luciferase assays and the presence of two HIF binding sites (HBS) (5'-GCGTG-3') termed pHRE1 and pHRE2 behaving as one unique HRE. Remarkably, although mutation of the 5' HRE in Kelly cells heavily reduced endogenous Epo mRNA and protein levels, no HIF binding at the 5' HRE was detected in Kelly wt cells, suggesting that the 5' HRE is functionally relevant for hypoxic *EPO* gene induction but not directly bound by HIF. This finding might be explained by the observation that some HIF target genes like, for instance, *PAG1* gene possess HREs to form chromatin loop locally or over a long distance to interact with the promoter without necessarily binding HIF but rather to keep a pre-formed chromatin loop ready to interact with other regulatory elements in case of hypoxic stimulus [10]. Therefore, we think that the 5' HRE might maintain a constitutive chromatin structure supporting the promoter activity in Kelly and, generally, in a cell-type specific manner. When generating the 5'/3' HREs double mutant Kelly clones, we obtained large and small 5' HRE deletions within the 3' HRE mutants. Intriguingly, large 5' HRE deletions fully abrogated endogenous Epo induction as well as binding of HIF-β at the *EPO* promoter region, indicating that the binding of putative transcription activators as well as HIFs is impaired. In contrast to large 5' HRE deletions, small 5' HRE deletions rather rescued Epo endogenous levels and HIF-β binding at *EPO* promoter, suggesting that additional transcription factors (TFs) might bind close to the consensus HRE sequence and be involved in chromatin looping and *trans*-activation of *EPO* promoter. Because single 5' HRE deletions strongly reduced Epo endogenous mRNA and protein levels as well as HIF-β binding at *EPO* promoter, it is currently unknown why small 5' HRE deletions in 3' HRE mutants rescue instead endogenous Epo levels and the interaction of HIF to the promoter. A scheme summarizing the proposed molecular mechanism of Epo transcriptional regulation in Kelly cells is displayed in Figure 1.

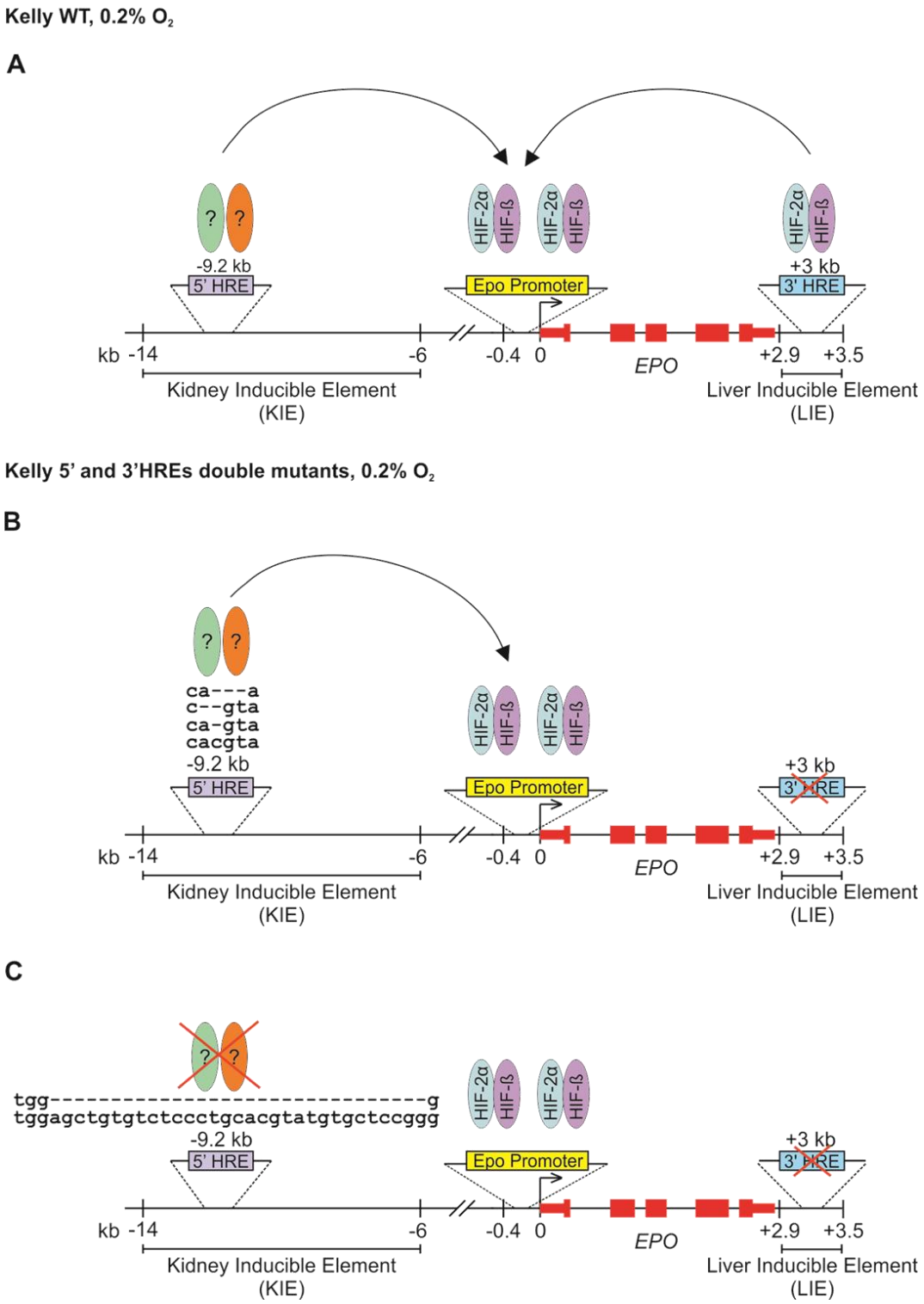

Figure 1. Proposed molecular mechanism of Epo transcriptional regulation in Kelly neuroblastoma cells. A. In wt cells, the heterodimer HIF-2α/HIF-β binds the 3' HRE as well as the two promoter HREs, whereas unknown transcriptional factors (TFs) bind the 5' HRE. A complex interplay between the 3' HRE looping to Epo promoter (black arrow on the right) and the 5' HRE looping to the Epo promoter (black arrow on the left) enhance Epo transcription up to 100-fold in hypoxia. B-C. In 5'/3' HREs double mutants, the interaction between the distal 3' HRE and the Epo promoter is impaired. While in double mutants with small 5' HRE deletions (1, 2 or 3 bp as indicated in B) unknown TFs can still bind the neighbour HRE consensus core ensuring the interaction with Epo promoter (black arrow on the left), a big deletion within the 5' HRE (31 bp as indicated in C) impedes the binding of any putative TFs, blocking the looping to Epo promoter.

These new findings in neuroblastoma cells shed light, for the first time, on the molecular mechanisms underlying the oxygen-regulated Epo transcription regulation in the brain. Although, Epo transcriptional regulation in the kidney remains elusive, the observation that REP cells express neuronal markers [11] renders the Kelly cells a better model than hepatoma cells to recapitulate human hypoxic *EPO* gene expression in the kidney. A recent study reported that transgenic mice having a deletion of the region -17.4 to 3.6 kb upstream of *EPO* showed abrogation of Epo expression in REP cells but not in brain, indicating that such region contained regulatory elements relevant exclusively for the kidney. However, deletion of the 0.7 kb sequence containing the mouse -8.3 kb 5' HRE (corresponding the human -9.2 kb 5' HRE) within this Epo 5' upstream region did not abrogate transgenic GFP expression in the kidney whereas the brain was not evaluated. In contrast, a minimal 0.3 kb fragment encompassing the mouse 5' HRE was insufficient to drive transgenic GFP expression in mouse kidney (again the brain was not analysed) [12]. Altogether, our findings on the complex interplay between the distal 5' and 3' HREs and *EPO* promoter in Kelly cells could help to understand *in vivo* renal Epo regulation: in the kidney Epo transcription might be regulated by the cooperative action of an extended 5' element, the minimal 3' HRE and the Epo promoter. To test this hypothesis it would be useful to generate transgenic mice with deletion of either the 0.7 kb sequence containing the mouse -8.3 kb 5' HRE or the 3' HRE and verify transgenic GFP expression not only in kidney but also in brain in order to confirm our findings about brain Epo regulation *in vivo*. In case the combined deletion of the 0.7 kb sequence and the 3' HRE would not be sufficient to abrogate the reporter gene expression, it would be still worth to delete combinations of extended 5' sequences as the region -17.4 to 3.6 kb upstream of *EPO* has been proven to be involved in renal Epo regulation.

In the future, to prove that the distal 5' and 3' HREs loop to Epo promoter and that the HIF-independent 5' HRE contain a pre-formed chromatin loop, it would be necessary to perform a chromosome conformation capture (3C). Additionally, to validate the contribution of the Epo promoter to endogenous Epo expression in Kelly, it would be worth to mutate Epo promoter sequence by CRISPR/Cas9. However, because the two newly reported promoter HREs, pHRE1 and pHRE2, are separated by only 29 bp, it will be difficult to target separately such HREs by sgRNA. On the other hand, in reporter gene assays pHRE1 and pHRE2 behave as one unique HRE, therefore, it would be still useful to target both promoter HREs by CRISPR/Cas9. Another important aspect to follow up is the identification of the TFs binding the 5' and 3' HRE in neuroblastoma in order to define the details of Epo transcriptional regulation in brain. As an example, predicted binding sites of putative TFs binding the enigmatic 5' HRE through *in silico* analysis by JASPAR database are depicted in Figure 2. Currently, our collaborators are employing a 5' HRE-dependent reporter gene assay to

screen a library of TFs to identify possible factors binding the novel 5' HRE. Multiple candidates have been found to enhance 5' HRE-driven luciferase activity and evaluation of Epo endogenous expression in shRNA interference conditions is ongoing.

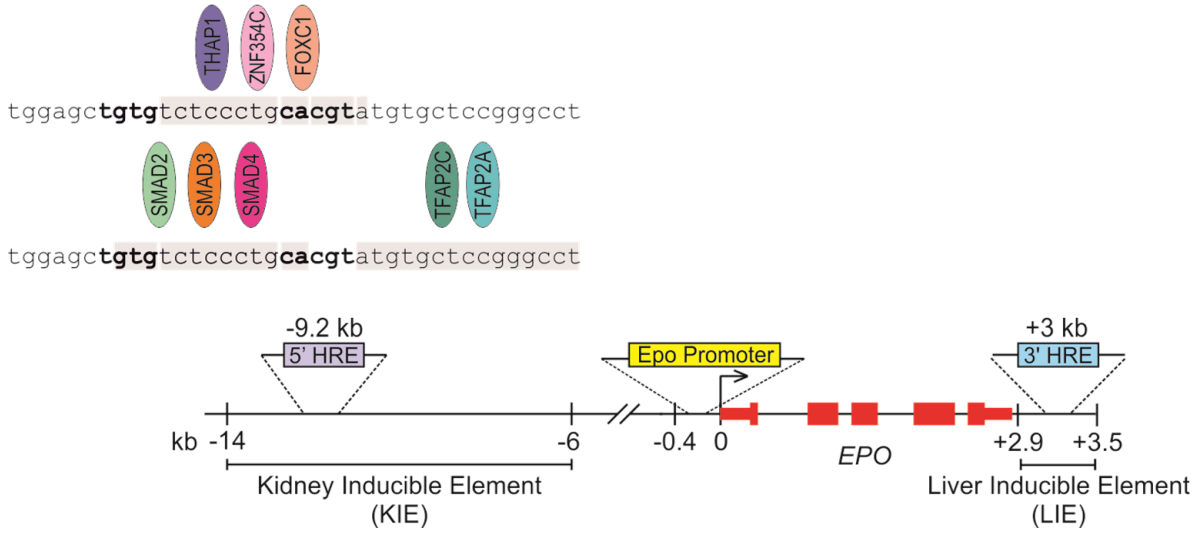

Figure 2. Predicted binding sites for putative TFs at the 5' HRE by JASPAR database. The predicted binding sites within the 5' HRE region are highlighted in grey, whereas the CACA element and the HRE core consensus ACGTG are showed in the reverse strand orientation and highlighted in bold. Putative TFs include small mother against decapentaplegic 2/3/4 (SMAD2/3/4), transcription factor AP-2 alpha/gamma (TFAP2A/C), THAP domain-containing protein 1 (THAP1), Zinc Finger Protein 354C (ZNF354C), Forkhead Box C1 (FOXC1).

Additionally, a strategic approach that could be considered in the near future, once one or more TFs binding the 5' HRE will be defined, is the pull-down of unknown proteins binding the 5' HRE using as bait the putative TF, followed by Mass Spectrometry analysis to discover more interactors at the 5' HRE. It would be worth to adopt this method in both Kelly neuroblastoma and in our non-transformed renal FAIK cell line as TFs binding the 5' HRE might be tissue-specific and, thus, different between brain and kidney. As mentioned before, methylation of CpG sites is an additional layer of regulation of Epo transcription; therefore, it would be interesting to evaluate the methylation status of the novel 5' HRE in hepatoma and neuroblastoma. In fact, methylated 5' HRE would explain its dispensable role in hepatic cells, whereas un-methylated 5' HRE would be expected in Kelly cells consistent with its important role in regulating endogenous Epo. Furthermore, we would like to repeat the CRISPR/Cas9 mutation of 5' and 3' HREs as well as Epo promoter in FAIK cells in order to decipher the contribution of these regulatory elements to renal Epo expression. Finally, as already mentioned in the unpublished data section, we would like to verify whether the HIF-dependent (3' HRE) and the HIF-independent (5' HRE) enhancers influence the cell-to-cell

variability of the Epo hypoxic transcript pattern by applying the ISH method to 5' and 3' HREs single and double mutant Kelly cells.

Final remarks

The transcriptional regulation of Epo in the kidney remains the main open question within the Epo research field and we hope to contribute soon in deciphering such mechanisms by performing the enhancers and promoter mutation analysis in our recently generated non-transformed renal FAIK cell lines, benefitting from the new discovered roles of 5' and 3' HREs in Kelly neuroblastoma cells.

Investigating the regulation of erythropoietin is important not only from a physiological prospective but, especially, for its clinical implications. Up-regulation of Epo levels causes, indeed, erythrocytosis whereas low Epo levels provoke anemia or anemia associated with chronic kidney disease (CKD). Nowadays, the therapy employed to cure patients affected by CKD relies on the injection of human recombinant Epo (rhEpo) that is, however, expensive. Therefore, PHD inhibitors have been developed and are currently in Clinical Trial III phase as possible substitute of rhEpo, being cheaper than rhEpo and orally administered. These drugs will enter the market in 1-2 years for the treatment of renal anemia but their mechanism of action needs to be further analysed as well as their potential side effects. Within such scenario, identifying the DNA regulatory elements involved in Epo transcriptional regulation opens the possibility to discover the TFs binding at such regulatory sites that, in the future, may be employed as novel drugs to enhance the endogenous levels of Epo, being smaller and cheaper than rhEpo and able to reduce the therapy costs and the side effects of patients affected by CKD.

References

1. Cassavaugh, J. and K.M. Lounsbury, *Hypoxia-mediated biological control.* J Cell Biochem, 2011. **112**(3): p. 735-44.

2. Wang, G.L. and G.L. Semenza, *Purification and characterization of hypoxia-inducible factor 1.* J Biol Chem, 1995. **270**(3): p. 1230-7.

3. Jelkmann, W., *Regulation of Erythropoietin production.* J Physiolo, 2011.

4. Suzuki, N., et al., *Specific contribution of the erythropoietin gene 3' enhancer to hepatic erythropoiesis after late embryonic stages.* Mol Cell Biol, 2011. **31**(18): p. 3896-905.

5. Kochling, J., P.T. Curtin, and A. Madan, *Regulation of human erythropoietin gene induction by upstream flanking sequences in transgenic mice.* Br J Haematol, 1998. **103**(4): p. 960-8.

6. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5680-4.

7. Wenger, R.H. and A. Kurtz, *Erythropoietin.* Compr Physiol, 2011. **1**(4): p. 1759-94.

8. Storti, F., et al., *A novel distal upstream hypoxia response element regulating oxygen-dependent erythropoietin gene expression.* Haematologica, 2014. **99**(4): p. e45-8.

9. Stolze, I., et al., *Hypoxia-inducible erythropoietin gene expression in human neuroblastoma cells.* Blood, 2002. **100**(7): p. 2623-8.

10. Schorg, A., et al., *Destruction of a distal hypoxia response element abolishes trans-activation of the PAG1 gene mediated by HIF-independent chromatin looping.* Nucleic Acids Res, 2015. **43**(12): p. 5810-23.

11. Imeri, F., et al., *Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation, and a telocyte phenotype.* Kidney Int, 2019. **95**(2): p. 375-387.

12. Hirano, I., et al., *Renal Anemia Model Mouse Established by Transgenic Rescue with an Erythropoietin Gene Lacking Kidney-Specific Regulatory Elements.* Mol Cell Biol, 2017. **37**(4).

7. Own contributions to the thesis

Manuscript I: Distal and proximal hypoxia response elements cooperate to regulate organ-specific erythropoietin gene expression

Ilaria M. C. Orlando, Véronique N. Lafleur, Federica Storti, Patrick Spielmann, Lisa Crowther, Sara Santambrogio, Johannes Schödel, David Hoogewijs, David R. Mole, and Roland H. Wenger

All figures except figure 4 B-C-D, figure 5 A-B and supplementary figure 4.

Manuscript II: Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation and a telocyte phenotype

Faik Imeri, Karen A. Nolan, Andreas Bapst, Sara Santambrogio, Irene Abreu Rodríguez, Patrick Spielmann, Svende Pfundstein, Silvana Libertini, Lisa Crowther, **Ilaria M. C. Orlando**, Sophie L. Dahl, Anna Keodara, Willy Kuo, Vartan Kurtcuoglu, Carsten C. Scholz, Weihong Qi, Edith Hummler, David Hoogewijs and Roland H. Wenger

Contributed to perform mEpo RT-qPCR, test Epo antibodies for immunoblotting, optimize transfection efficiency in FAIK cells.

Unpublished results: single cell variability of hypoxia-inducible gene expression

Ilaria M.C. Orlando, Patrick Spielmann and Roland H. Wenger

All figures.

8. Curriculum Vitae

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Education

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Publications

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Imeri F., Nolan K. A., Bapst A. M., Santambrogio S., Abreu Rodriguez I., Spielmann P., Pfundstein S., Libertini S., Crowther L., **Ilaria M. C. Orlando** et al. Generation of renal Epo-producing cell lines by conditional gene tagging reveals rapid HIF-2 driven Epo kinetics, cell autonomous feedback regulation and a telocyte phenotype. *Kidney Int. 2018*

Conferences and presentations

Oral presentations

09/2015	Short blitz presentation (SPS) at the Annual Swiss Physiological Society Meeting; Bern, Switzerland
09/2016	Short blitz presentation (SPS) at the Annual Swiss Physiological Society Meeting; Fribourg, Switzerland
02/2017	LS^2 Life Science Switzerland Annual Meeting 2017; Zurich, Switzerland
02/2017	NCCR Kidney.CH Retreat in Murten; Switzerland
09/2017	13th Symposium of the Zurich Center for Integrative Human Physiology; Zurich, Switzerland
04/2018	Keystone Hypoxia Meeting; Oxford, UK Therapeutic Targeting of Hypoxia-Sensitive Pathways

Poster presentations

08/2015	11th Symposium of the Zurich Center for Integrative Human Physiology; Zurich, Switzerland
02/2016	NCCR Kidney.CH Retreat in Murten; Switzerland

08/2016	12th Symposium of the Zurich Center for Integrative Human Physiology; Zurich, Switzerland
03/2017	Keystone Symposia Meeting; Whistler, BC, Canada Adaptations to Hypoxia in Physiology and Disease
09/2017	Annual Swiss Physiological Society Meeting; Bern, Switzerland
02/2018	NCCR Kidney.CH Retreat in Murten; Switzerland
02/2019	NCCR Kidney.CH Retreat in Murten; Switzerland

Awards

09/2016	Young Investigator Award “Oetliker Prize” for the best poster presentation at the Physiology Meeting 2016 in Fribourg, Switzerland
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